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CULTURE OF LOUPING-ILL VIRUS IN THE EMBRYONATED EGG.

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MULTIPLICATION of louping-ill virus in the egg was first demonstrated by Burnet (1936). He adapted a strain to grow on the chorio-allantoic membrane and used a count of the focal lesions which resulted from the inoculation of suitable dilutions as a method for titrating the virus. Growth in the chick embryo was not otherwise studied. The increasing use of culture in the embryonated egg to obtain suspensions of viruses suitable for use as antigens suggested a further investigation of the growth of louping-ill virus in the chick embryo.

Technical Methods.

Two strains of louping-ill virus were obtained from the Animal Diseases Research Association, Moredun, through the kindness of Dr. D. R. Wilson. One, a labroatory strain, received as dried infected sheep brain, was passaged serially intracerebrally in mice for up to six passes before being inoculated into chick embryos. The other strain was used to inoculate eggs after only two or three passages in mice, subsequent to its isolation from a sheep infected in the field.

For incubation of the fertile eggs prior to inoculation a temperature of 37·8° C. was aimed at, though in the absence of a suitable incubator it was not possible to obtain critical control over this temperature. After inoculation the eggs were incubated at 35° C. Inoculations of 0·1 ml. into the yolk sac were made by the usual technique (Beveridge and Burnet, 1946). The site of inoculation was dabbed with hot melted paraffin wax before introduction of the needle and the hole was afterwards closed by applying more wax. Blind inoculations into the embryo itself, or its immediate neighbourhood, were also made. The egg was candled and the position of the embryo was marked; 0·1 ml. of inoculum was injected after introducing the needle for 2 cm. into the position of the embryo. Chorio-allantoic, allantoic and amniotic inoculations were performed by the usual techniques (Beveridge and Burnet, 1946).

Eggs were candled daily; embryos dying within 24 hours after inoculation were disregarded. It was usual to discard all dead embryos; eggs with living embryos when due for harvesting were chilled for from one to three hours before being opened. Suspensions (10 per cent) of the embryo, excluding the eyes,

were made in 10 per cent horse serum broth at pH 7·4, using either a Ten Broeck grinder for small amounts of tissue, or a mechanically-driven tissue fragmenter for larger amounts. Two types of the latter were employed, one a turbo-reactor and the other an instrument designed on the principle of the Waring Blendor. The supernatants were used after centrifuging for 3 minutes at 1500 r.p.m. For preservation of the virus infected tissues were stored in 50 per cent glycerine-saline at 0° C.

Virus was titrated by inoculating 0·025 ml. amounts of falling tenfold dilutions intracerebrally into groups of three or more young Swiss mice. The mice were kept under observation for three weeks. All deaths were noted. Usually animals that died had been observed previously to show the typical paralytic signs of louping-ill. The titres of virus were estimated by using a 50 per cent death rate as the end point, calculated by the method of Reed and Muench (1938). In recording the results the original 10 per cent suspension was regarded as 10^{-1}. Originally nutrient broth was used as diluent for suspensions of infected tissues, but subsequently 10 per cent horse serum broth was found superior. Dilutions of an infected sheep brain in saline, tested immediately and again after standing three hours on the bench, showed a fall in titre from $10^{-5.5}$ to $10^{-3.5}$. Dilutions made in nutrient broth fell in titre over that period from $10^{-5.5}$ to $10^{-4.2}$; dilutions in serum broth did not alter in titre. When 10 per cent suspensions in these three diluents were titrated after storage for 24 hours at +4° C. in saline and plain broth, the titre fell from $10^{-5.5}$ to $10^{-4.2}$; in serum broth the titre was unaffected.

Passage of virus in the egg.

The first attempts to adapt louping-ill virus to the fertile egg were made by inoculating infective mouse brain suspensions onto the chorio-allantoic membrane and into the allantoic sac; serial passages were made with suspensions of the membrane and with the allantoic fluid. Virus disappeared after a few passes, and daily titration showed no multiplication in the allantoic fluid. Embryos were successfully infected by inoculating the eggs directly into the region of the embryo. Serial passage was obtained by inoculating fresh batches of eggs with this technique using suspensions of the infected embryos. One strain was maintained for 64 serial passes in the embryonated egg. Subsequently it was found that inoculation into the yolk sac was as good, or even better, for initiating and maintaining passage.

Daily titrations after inoculation suggested that the titre of virus in the embryo was highest on the third day. Usually by then in a batch of 12 eggs several embryos had died. Therefore for the first 11 passes embryos were harvested for passage on the third day. When it was noted that no embryos were dying on the third day, the procedure was changed and living embryos were harvested on the day when about a third of the embryos in a batch had died. This death rate was usually obtained on the fifth or sixth day, but was dependent on the route of inoculation and the age of the embryos at the time of inoculation. Subsequent experiments suggested advantages in using yolk sac inoculation of eggs that had been incubated for 6 days, and this became the routine practice. Embryos were then found to be dying earlier and from about the 24th to the 51st pass, in order to increase the number of passes that could be made in a given time, embryos were harvested on the second or third day, irrespective of the death rate among

the embryos. In later passes a third of 6-day embryos inoculated into the yolk sac were usually dead by the third day and all were dead by the sixth day ; 10-day embryos survived about one day longer. Throughout these experiments the suspensions used for passage were made from living embryos, either immediately after harvesting, or after they had been stored in the cold for a short period in 50 per cent glycerine-saline.

The macroscopic changes produced in the infected egg were striking, although not characteristic. The changes were best seen in eggs that had been inoculated at 10 days' incubation and had been harvested just before death of the embryo. The embryos were always markedly soft and oedematous. Oedema was commonly accompanied by a gelatinous degeneration of the tissues, and this often caused considerable swelling, particularly of the abdomen. Haemorrhages, varying from a few small haemorrhages under the skin to others much larger and diffuse, were common. Like the other tissues the brains were markedly soft, swollen and oedematous, and sometimes contained minute haemorrhages. The chorio-allantoic membranes, whether they had been inoculated directly or had been infected by inoculation into the yolk sac, were regularly greatly swollen from oedema and gelatinous degeneration. Embryos were usually pale, due to oedema, but neither anaemia nor jaundice, such as were described by Burnet (1936), were noted to result from infection by these strains of virus.

During its propagation in eggs the titre of virus in embryo suspensions was regularly estimated by the intracerebral inoculation of mice. Titres ranged between $10^{-5.5}$ and $10^{-7.5}$. Numerous experiments were performed to determine the optimum conditions for multiplication of the virus in the embryo.

Rate of multiplication of virus in the embryo.

At the second passage in eggs a batch of 10-day embryos were inoculated intra-embryonically. Daily from the first to fourth days after inoculation pairs of living embryos were harvested, pooled and the virus titrated. On the first day there was a titre of $10^{-2.5}$, on the second $10^{-5.5}$, on the third $10^{-6.3}$, and on the fourth $10^{-5.3}$. This experiment was repeated at the eighth passage, groups of three embryos chosen at random being pooled for titration of virus. The titre of virus increased progressively from $10^{-2.8}$ on the 1st day to $10^{-6.0}$ on the fourth day and was even higher, at $10^{-7.0}$, on the sixth. Subsequent similar experiments using 6- and 8-day embryos inoculated into the yolk sac also gave variable results. It seemed probable that most virus was present shortly before death of the embryo, and it was found more convenient to use the death of a moderate proportion of the embryos in a batch as the indication for harvesting the rest of the batch. Dead embryos, harvested within 24 hours of death, always had a lower virus titre than living embryos and were therefore discarded.

Effect of age of embryo and method of inoculation.

Numerous experiments were undertaken to discover the optimum age of the embryo and the best method of inoculation. Deaths were fewer and titre of virus lower in 14- to 16-day than in 11-day embryos inoculated by the intra-embryonic technique. For the infection of younger embryos inoculation into the yolk sac was preferred. It is likely that when 8-day embryos were injected blindly into the region of the embryo the inoculum often entered the yolk sac instead of the body of the embryo. The results of several comparative titrations are summarized

in Table I ; in these 6-, 8- and 10-day embryos were inoculated into the yolk sac, by the intra-embryonic technique, and on to the chorio-allantoic membrane. The size of the inoculum, 0·1 ml., was the same for each method of inoculation. By the 10th day the yolk sac has much decreased in size, and it is possible that in a proportion of eggs of that age the inoculum did not reach the yolk sac. Living eggs were harvested when a third of the embryos in the batch had died ; they were pooled and suspensions made under similar conditions for titration of virus. Sometimes the death rate was considerable and few embryos survived for harvesting. It is possible that this eliminated the more susceptible embryos, and may have helped to account for the considerable variation in results from experiment to experiment.

In these experiments both white and brown leghorn eggs were used. Comparative experiments in which batches of eggs of the two breeds were inoculated separately with similar inocula showed them to be equally sensitive to the virus ; the titres attained in each were the same.

Distribution of virus in the infected egg.

Virus in the embryo, chorio-allantoic membrane, yolk sac, amniotic and allantoic fluids was titrated after various methods of inoculation. Serial dilutions were made directly from the fluids, but with the other materials 10 per cent suspensions in serum broth were first made using Ten Broeck grinders. In calculating the results the 10 per cent suspensions were regarded as 10^{-1}. The results (Table II) showed that most virus was to be found in the embryo itself. There was only a low titre of virus in the yolk sac, even when inoculation had been made directly into it. Considerable amounts of virus were present in the chorio-allantoic membrane ; after inoculation directly on to the membrane there was even a higher titre of virus in the membrane than in the embryo. The amniotic fluid had a high titre only after intra-embryonic inoculation ; probably with this method the inoculum was often injected directly into the amniotic cavity. The higher titre in the amniotic fluid than in the embryo in this experiment was exceptional ; in other experiments the titre was usually less. The allantoic fluid contained less virus than the amniotic fluid.

The extra-embryonic fluids which are free of tissue fragments and contain relatively small amounts of non-specific protein are to be preferred for obtaining suspensions of virus. The growth of louping-ill virus in the amniotic and allantoic cavities was therefore investigated further. Before adaption to growth in the egg virus multiplied little if at all when inoculated into the allantoic cavity. After adaptation, using 32nd or later passage virus as inoculum, considerable amounts of virus were found following allantoic inoculation of 8 to 12 day eggs. However the maximum titre ($10^{-5\cdot2}$) obtained in these experiments was not sufficiently high to encourage further use of this method. Titres about ten times higher than this were obtained in the amniotic fluid after direct inoculation into the amniotic cavity. There was an equally high titre of virus in the amniotic fluid after blind inoculation into the region of the embryo, and there appeared to be no advantage in using the more difficult technique.

Effect of amount of virus in the inoculum.

With certain viruses it has been found that the maximum titres of virus obtained by culture in the egg depended on the concentration of virus in the

TABLE I.—*Effect of Method of Inoculation and Age of Embryo.*

Number of serial passes in eggs.	Age of embryo and method of inoculation.																	
	6-day embryos into yolk sac.			8-day embryos into yolk sac.			10-day embryos into yolk sac.			10-day embryos into embryo.			10-day embryos on to chorio-allantoic membrane.		
	Day of harvesting.	Mortality ratio.	Virus titre.	Day of harvesting.	Mortality ratio.	Virus titre.	Day of harvesting.	Mortality ratio.	Virus titre.	Day of harvesting.	Mortality ratio.	Virus titre.	Day of harvesting.	Mortality ratio.	Virus titre.
12	—	—	—	5	6/14	$10^{-6.8}$	—	—	—	7	8/14	$10^{-5.5}$	4	4/14	$10^{-5.8}$
13	5	5/15	$10^{-6.8}$	6	10/15	$10^{-5.5}$	6	9/15	$10^{-6.5}$	6	13/17	$10^{-5.0}$	—	—	—
15	4	4/10	$10^{-5.5}$	5	7/18	$10^{-6.5}$	—	—	—	—	—	—	—	—	—
19	4	15/26	$10^{-6.15}$	4	14/32	$10^{-5.80}$	5	10/20	$10^{-6.15}$	6	11/19	$10^{-5.63}$	—	—	—
39	—	—	—	4	4/9	$10^{-6.0}$	4	2/6	$10^{-6.8}$	4	1/6	$10^{-6.5}$	—	—	—

The inoculum throughout was 0·1 ml. of a 10 per cent embryo suspension. — signifies that no observation was made.

TABLE II.—*Distribution of Virus in Eggs Inoculated by Different Routes.*

Number of serial passes.	Age of embryo in days.	Route of inoculation.	Titre of virus.				
			Embryo.	Chorio-allantoic membrane.	Yolk sac.	Amniotic fluid.	Allantoic fluid.
8	10	Into embryo	$10^{-5.2}$	$10^{-4.8}$	$10^{-3.8}$	$10^{-6.2}$	$10^{-4.5}$
8	10	On to chorio-allantoic membrane	$10^{-4.5}$	$10^{-6.2}$	—	—	—
8	8	Into yolk sac	$10^{-5.5}$	$10^{-3.8}$	$>10^{-3.0}$	$10^{-4.2}$	$10^{-3.8}$
18	6	Into yolk sac	$10^{-6.28}$	$10^{-5.28}$	$10^{-4.75}$	$10^{-4.2}$ (or less)	$10^{-3.63}$

0·1 ml. of a 10 per cent embryo suspension inoculated. — signifies that no observation was made.

inoculum. Higher titres of influenza virus were obtained in the allantoic fluid when the inoculum was diluted by 10^{-2} or 10^{-3} than with a less diluted inoculum (Miller, 1944). On the other hand the highest titres of Japanese B encephalitis virus in the chick embryo were obtained by inoculating suspensions of infected mouse brain which had titres higher than could be obtained in the embryo itself (Howitt, 1946). The effect of the concentration of virus in the inoculum was studied for louping-ill. When an undiluted embryo suspension and a tenfold dilution of this were inoculated into batches of eggs, using intra-embryonic inoculation, there was no significant difference in titre of virus in the pooled embryos from the two batches, when harvested on the third day. With a hundred-fold dilution the titre was reduced, but possibly not significantly. Following inoculation with a thousand-fold dilution the titre of virus was much reduced.

A recently isolated strain of virus after only two passages in mice was inoculated into eggs using yolk sac inoculation of 6-day embryos. Serial passage in the eggs was maintained for 6 passes, but the yields of virus in the embryos were no higher than with the original strain of virus. This strain, however, gave very high titres ($10^{-8.5}$) in the mouse brain. When 10 per cent suspensions of these brains containing a high concentration of virus were inoculated into eggs, the titres of virus reached in the embryo were no higher than with the other strain.

Titration of virus in the chick embryo.

Burnet (1936) and Burnet and Lush (1938) titrated suspensions of louping-ill virus by inoculating serial dilutions on to the chorio-allantoic membrane and counting the number of focal lesions produced. More recently Koprowski and his collaborators titrated in eggs two other neurotropic viruses, Japanese B and Venezuelan equine encephalomyelitis, by estimating the dilution which would kill 50 per cent of the embryos (Koprowski and Cox, 1946, Koprowski and Lennette, 1946*a*). It has been possible to use the death rate among chick embryos for titrating louping-ill virus.

At first serial dilutions were inoculated directly into 10-day embryos. The titrations were not satisfactory, as a number of embryos survived inoculation with the low dilutions. A more regular response was obtained by using the yolk sac inoculation of 8-day embryos. Results were not improved by using 6-day embryos. Deaths within 24 hours after inoculation were disregarded. Thereafter eggs were candled daily and deaths recorded. Experiments terminated on the 20th day after the commencement of incubation.

Comparative titrations of virus in the brain of an infected sheep, made by the yolk sac inoculation of eggs and by the intracerebral inoculation of mice, showed the former to be a more sensitive method ; the titre was a hundred times higher than with the latter method (Table III). In several comparative titrations of the egg-adapted virus the inoculation of the chick embryo gave a titre at least

TABLE III.—*Titration of an Infected Sheep Brain in Chick Embryos and in Mice.*

Method of inoculation.	Volume of inoculum in ml.	Mortality ratios for dilution of virus.					Estimated $L.D._{50}$ titre.
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
Into yolk sac of 8-day embryos	0·1	4/4	4/5	4/5	5/5	0/4	$10^{-6.30}$
Intracerebral in mice	0·025	4/5	3/5	1/5	0/6	—	$10^{-5.16}$

10 times that given by intracerebral inoculation of mice. The inoculum for the embryos (0·1 ml.) was 4 times that for the mouse. The greater sensitivity of the chick embryo for detecting certain viruses has been shown previously. Burnet (1936) found that the counting of focal lesions on the chorio-allantoic membrane was 10 times more sensitive than the inoculation of mice for detecting louping-ill virus. Howitt (1946), who titrated Japanese B encephalitis in the chick embryo by estimating the death rate, obtained titres 10 times greater than those given by inoculation of mice.

This method for detecting virus was also used in serum neutralization tests. A series of falling tenfold dilutions of the 37th egg passage virus in horse serum broth were mixed with equal volumes of the serum of a sheep hyper-immunized against louping-ill. A similar series of dilutions of virus mixed with normal horse serum were set up as controls. Both sets of mixtures were kept for two hours at room temperature, followed by one hour in the refrigerator (Rivers and Schwentker, 1934) and then tested for live virus by the inoculation of 8-day embryos into the yolk sac (Table IV). Previous experiments carried out similarly,

TABLE IV.—*Neutralization of 37th Embryo Passage Virus using the Yolk Sac Inoculation of 8-day Embryo as a Test for Live Virus.*

Inoculum.	Mortality ratio of embryos for dilutions of virus.							
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Virus dilution and immune sheep serum	4/4	3/4	2/4	1/3	0/3	—	—	—
Virus dilution and control horse serum	—	—	4/4	4/4	3/4	0/4	2/4	0/3

except that infected mouse brain was used as the source of virus and that the dilutions were tested for virus by intracerebral inoculation of mice, had shown the immune serum to be capable of neutralizing between 100 and 1000 M.L.D. of virus. The neutralization test carried out in eggs gave a similar result. It thus demonstrated that the virus present in the 37th pass in eggs was still serologically identical with louping-ill virus.

Pathogenicity of the embryo-passage virus.

During its passage in chick embryos the strain of louping-ill virus was examined for alterations in its pathogenicity for animals and chick embryos. By the 64th passage the virus did not appear to have become more lethal for the embryo than unadapted virus, and there had been no significant increase in the concentration of virus in the embryos.

An embryo suspension at the 49th passage was used for comparative titrations

TABLE V.—*Comparative Titrations of 49th Embryo Passage Virus in Chick Embryo, Mice and Sheep.*

Method of titration.	Volume of inoculum in ml.	Mortality ratios for dilutions of virus.								Estimated $L.D._{50}$ titre.
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	
Into yolk sac of 8-day embryos	0·1	5/5	4/4	4/5	5/5	2/5	2/5	2/5	0/4	$10^{-8.5}$
Intracerebral in mice	0·025	5/5	4/5	1/5	1/5	0/5	—	—	—	$10^{-6.6}$
Intracerebral in sheep	1·0	2/2	2/2	2/2	0/2	0/2	—	—	—	$10^{-6.5}$ (approx.)

in eggs, mice and sheep (Table V). The sheep received 40 times the inoculum used for the mice, and this probably accounted for the difference in titres obtained by inoculation of sheep and mice. Two sheep only were inoculated with each dilution, and thus the $L.D._{50}$ for sheep was only approximate. Use of the 50 per cent embryo death rate as a means for estimating titre gave a result nearly a thousand times greater. It has already been shown (Table III) that this method of titration in eggs was 100 times more sensitive for demonstrating sheep brain virus. The fact that after adaptation to the egg the difference between the titres given by the two methods had increased tenfold may mean that the virus had increased in virulence for the embryos, relative to its virulence for the mouse. However, the difference is not great and may not be significant. In other comparative titrations on earlier passage material such a difference was not noted. At any rate it appeared that throughout its passage in eggs the strain had remained neurotropic, and could produce a fatal infection when injected directly into the brains of sheep and mice.

A 1 per cent suspension of embryos harvested at the 49th passage was inoculated intraperitoneally in 0·25 ml. amounts into mice; 7 out of 10 mice died. This mortality was as high as that obtained following intraperitoneal injection of mouse brain virus. Thus the egg passage virus had not lost its ability in the mouse to infect the brain after inoculation peripherally. On the other hand three sheep inoculated subcutaneously with 5 ml. of a 10 per cent suspension of the same material remained well and apyrexial for three weeks. They were then inoculated subcutaneously with 5 ml. of a highly infective sheep brain suspension, followed three days later by an intracerebral injection of starch. The animals showed no evidence of infection. In experiments, which will be reported subsequently, it was shown that in non-immune sheep, inoculated subcutaneously with louping-ill virus, the intracerebral injection of starch three days later caused a fatal encephalitis in 100 per cent of animals. When starch was not injected in about half the sheep involvement of the brain and death did not occur and the only evidence of infection was, possibly, a pyrexial response.

In a subsequent experiment 10 sheep were inoculated subcutaneously with 55th embryo passage virus; 5 ml. of a 1 per cent suspension was given to 5 animals and 5 ml. of a 0·1 per cent. suspension to the others. One animal died on the 16th day and showed post-mortem evidence of a generalized bacterial infection; virus was not recovered from its brain or spleen by the intracerebral inoculation of mice. Death therefore appeared to be due to non-specific causes. The remaining sheep showed no signs of illness although the temperatures of 5 were somewhat raised during the second or third weeks after inoculation. At the end of 3 weeks the sheep were challenged with a 10 per cent suspension of sheep brain virus. Two sheep inoculated previously with 1 per cent embryo suspension, and two with 0·1 per cent suspension, were injected intracerebrally; all 4 died of louping-ill. The other animals were challenged by subcutaneous inoculation of virus with an intracerebral injection of starch 3 days later. One animal, which had originally had 1 per cent embryo virus, died of louping-ill; the others were unaffected. Thus in all except one sheep subcutaneous inoculation with embryo virus produced immunity in surviving animals against sheep brain virus injected subcutaneously, but not against the same virus given intracerebrally. The one failure may have been due to challenge too soon after the original inoculation.

DISCUSSION.

Louping-ill virus was easily propagated in the embryonated egg by inoculating suspensions of infected embryos either into the yolk sac or blindly into the region of the embryo. The concentration of virus was highest in the embryo itself; in a large number of titrations the titre of virus varied between $10^{-5.5}$ and $10^{-7.5}$. It was not possible regularly to obtain suspensions with the highest titre by control of such factors as the breed of embryo, concentration of virus in the inoculum or time of harvesting. Likewise it was not possible to increase the titre by using high concentrations of virus in the inoculum. The variation in titre of different batches of embryos could not be accounted for and may have resulted from the influence of some uncontrolled factor; for instance it was not possible to obtain critical control of the temperature of pre-incubation. However, there were equal variations in titre between different batches of virus suspension obtained from infected sheep tissues.

Although the concentrations of louping-ill virus in the egg were not as high as those obtained with some other viruses, where embryo suspensions after inactivation have been effectively used as vaccines, the titres compared favourably with those obtainable in animal tissues. During large scale production of louping-ill virus from the brains, spinal cords and spleens of infected sheep for use as a vaccine the titre of virus varied between 10^{-4} and 10^{-6}. The concentration of virus was higher in the brains of infected mice, which had an average titre of 10^{-7}. The average titres were therefore very approximately 10^{-5} in sheep tissues, 10^{-6} in chick embryos and 10^{-7} in mouse brain. Wilson (1945), who cultivated louping-ill virus in tissue culture, obtained cultures infective for mice to limiting dilutions between 10^{-3} and 10^{-5}. The corresponding limits of infectivity for both amniotic fluids and 10 per cent embryo suspensions obtained by propagation in the embryonated egg were dilutions varying between 10^{-4} and $10^{-6.5}$.

There was no evidence of alteration in antigenicity resulting from passage in chick embryos. At the 37th passage virus was fully neutralized by a specific immune serum. Seven out of 8 sheep which had survived subcutaneous inoculation with 49th and 55th embryo passage virus were immune to subsequent subcutaneous injection with a strain of virus maintained by passage in sheep. After passage in chick embryos the virus remained neurotropic and fully virulent for sheep and mice when inoculated intracerebrally. Its ability to invade the central nervous system of mice after intraperitoneal injection was not appreciably affected. There was, however, evidence that after 49 passages pathogenicity for sheep had been modified. In 7 experiments carried out in the course of a year the original strain of virus maintained by intracerebral passage in sheep produced encephalitis and death in 11 out of 22 sheep inoculated subcutaneously. On the other hand 12 out of 13 sheep injected subcutaneously with the embryo adapted virus survived; the one death was probably due to non-specific causes. The susceptibility of sheep that had survived subcutaneous injection with embryo virus to intracerebral inoculation with sheep brain virus provided further evidence of a modification of the virus by passage in eggs. It was shown by Grieg *et al.* (1931) that sheep which have survived subcutaneous inoculation with potent virus, such as that in infected sheep brain, were resistant to a subsequent intracerebral inoculation with virus. It was recently found that the propagation of West Nile and Venezuelan equine encephalomyelitis viruses in tissue culture

produced a similar change in the pathogenic properties of each virus (Koprowski and Lennette, 1946*b*, 1946*c*). Although the viruses retained their neurotropic properties and were lethal at the same dilution as originally to mice injected intracerebrally, their ability to cause a fatal encephalitis when injected by other routes than directly into nervous tissues was progressively lost during passage. There is thus evidence that with these neurotropic viruses modification and lowered virulence, produced by propagation in an heterologous tissue, is shown by a diminished ability to invade the central nervous system from the blood. Wilson (1945), who cultivated louping-ill virus in tissue culture, noted that the virus at the 40th passage, when injected subcutaneously into sheep, although capable of causing a thermal response, failed to produce signs of involvement of the nervous system, or death, in the 10 animals injected. The sheep were resistant to subsequent challenge intracerebrally with sheep brain virus. It is possible that tissue culture had appreciably modified the virus, although Wilson did not investigate this further and regarded the thermal responses produced by the virus as evidence that it had retained its viscerotropic properties.

Further experiments in larger groups of sheep will be necessary to study the degree of attenuation produced in the embryo-adapted virus and to confirm its inability to invade the brain after subcutaneous inoculation. In view of the evidence that in sheep naturally infected in the field the co-existence of tick-borne fever helps to determine whether louping-ill virus invades the nervous system (Gordon *et al.*, 1932), there is the possibility that other infective or metabolic disorders in the sheep may affect the ability of virus to cause encephalitis.

SUMMARY.

Louping-ill virus was shown to multiply in the embryonated egg when inoculated into the yolk sac, or into the embryo itself, and was maintained for 64 passages. Embryos at 6 to 10 days' incubation were most susceptible. The yield of virus was not increased by using a diluted inoculum or one containing a particularly high concentration of virus.

The embryo itself contained the highest concentration of virus, although considerable amounts were present in the amniotic fluid following inoculation into the embryo or directly into the amniotic cavity. Embryo suspensions were infective for mice to a dilution between 10^{-5} and $10^{-7 \cdot 5}$. The highest titres of virus were found in embryos harvested shortly before death, which in the majority of embryos occurred three or four days after inoculation.

Infection of the embryo was fatal and estimation of the $L.D._{50}$ afforded a means of titrating virus about 100 times more sensitive than the intracerebral inoculation of mice.

Virulence after intracerebral inoculation of the embryo-adapted virus was not altered for sheep and mice. Ability to invade the nervous system from the blood was not appreciably affected for mice, but was apparently lost or considerably diminished for sheep. Subcutaneous inoculation of sheep protected 7 out of 8 animals against subsequent subcutaneous inoculation with virulent sheep virus, but did not protect against intracerebral injection of virus.

I wish to thank Mr. W. A. Fitzgerald for his valuable technical assistance throughout this investigation.

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INVESTIGATION INTO THE PRODUCTION OF BACTERIOSTATIC SUBSTANCES BY FUNGI. PRELIMINARY EXAMINATION OF THE SEVENTH 100 SPECIES, ALL BASIDIOMYCETES.

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THIS paper continues the series of publications which have appeared under the above title since 1942 (Wilkins, 1945, 1946*a*, *b*, 1947; Wilkins and Harris, 1942, 1943*a*, *b*, 1944*a*, *b*, *c*, *d*, *e*). Seven of these previous papers have each dealt with an examination of 100 species of various sorts of fungi, in order to determine to what extent they produce antibacterial substances. The last two of these seven papers and the present one deal solely with basidiomycetes, and represent a concentrated investigation of this group, which investigation is being continued. Reference to "the first" or "the second" previous papers means reference to the two previous papers on basidiomycetes only.

As mentioned previously, since the first paper appeared, Robbins, Hervey, Ross, Ma and Robbins (1945) have published an account of an investigation into the antibacterial properties of 300 basidiomycetes. The similarity of object and outlook in Robbins' paper and in mine invites comparison, and, through the courtesy of Professor Robbins, I am able to include here 66 species which I have received from him in exchange for some of my cultures. These American cultures are indicated by the Robbins' number, letter or both, in a bracket after the name of the fungus. The other cultures examined were obtained either from the Forest Products Research Laboratory, by the kindness of the Director, or were purchased from the Centraalbureau voor Schimmelcultures, Baarn, Holland.

The former are indicated by the Laboratory Number, not in a bracket, while the latter have the letters CBS after the name of the fungus.

As before, the fungi were grown on malt or potato dextrose agar and tested initially by the "strip" method (Wilkins and Harris, 1944*b*). Those which gave a positive reaction to this test were then grown on malt and potato dextrose liquid media, and the metabolism solution was tested by the "hole" method (Wilkins and Harris, 1943*b*). Those species which were negative to the strip test were not tested further. In all cases the tests were made against the two representative types of bacteria, *Bacterium coli* and *Staphylococcus aureus*. The results are given in the following lists and are arranged in three classes: (1) "strongly positive," i.e. those fungi in which the diameter of the area of inhibition is 20 mm. or more; (2) "weakly positive," i.e. those in which the diameter is 15–20 mm.; and (3) "negative," i.e. those which gave no reaction in the initial strip test. Details as to the degree of inhibition as indicated above are given only for the first class, and here, in contrast to the two previous papers, results of the initial strip test, which is regarded merely as a positive/negative indication, are not included, and the results of the hole test are given for the most favourable liquid medium.

LIST OF FUNGI EXAMINED.

Strongly Positive.

	Result of test on liquid medium.			
	Time in days.	Medium.*	*Bact. coli.*	*Staph. aureus.*
Auricularia auricula-Judae (Bull. ex Fr.) Secretan. CBS	30	M.	17	20
Corticium abeuns Burt. (14572-R)	15	M.	—	23
C. hydnans (Schw.) Burt. (57407-R)	20	M.	18	22
Daedalea heteromorpha Fr. (59051-R) = *Trametes heteromorpha* (Fr.) Bres. & Neuman. = *Lenzites heteromorpha* Fr.	10	M.	—	25
Fomes fraxinophilus (Peck) Sacc. (59120-S)	25	M.	—	21
F. officinalis (Vill. ex Fr.) Neuman (80518-S)	30	M.	22	20
Ganoderma oregonense Murr. 111	25	M.	—	29
Hirneola sp. Fr. (Iowa 42)	5	M.	—	20
Hydnum abietis Weir. (3548)	20	M.	—	23
Irpex mollis B. & C. (41542-R)	10	P.D.	17	25
Lepiota naucina (Fr.) Quél. (312)	10	P.D.	23	26
Naucoria semiorbicularis (Bull. ex Fr.) Quél. CBS	20	M.	—	22
Pleurotus griseus Peck (71423-R)	15	M. or P.D.	—	20
Polyporus biformis Fr. & Kl. (71423-R) = *Polystictus biformis* (Fr. ex Kl.) Cooke.	10	M.	22	28
P. delectans Peck. (71423-R)	15	M. or P.D.	—	23
Radulum orbiculare Fr. (57402-S)	30	M.	15	20

* M. = malt; P.D. = potato dextrose.

Weakly Positive.

Boletus scaber Bull. ex Fr. CBS; *Claudopus nidulans* (Pers. ex Fr.) Karst. (71364-S); [= *Crepidotus nidulans* (Pers. ex Fr.) Quél.]; *Clitocybe aurantiaca* (Wulf. ex Fr.) Studer. CBS; *C. infundibuliformis* (Schaeff. ex Fr.) Quél. CBS; *C. robusta* Peck. CBS; *Coniophora suffocata* (Peck) Massee (56488-S); [= *C. sistremoides* (Schw.) Massee sec Burt.]; *Corticium conigenum* Shear (66211-Sp.); *Daedalea juniperina* (Murr.) P. Syd. (71585-R); *Echinodontium tinctorium* Ell. & Ev. 78; *Flammula alnicola* (Fr.) Quél. (2071); *F. purpurata* Cooke & Massee 310; *Fomes fraxinophilus* (Peck.) Sacc. (B); *F. pini* (Thore ex Fr.) Lloyd (52061-S); *F. subroseus* (Weir) Overholts. 301; [= *Trametes subrosea* Weir.]; *F. ungulatus* (Schaeff.) Sacc. CBS; [= *F. marinatus* (Fr.) Gill. = *F. pinicola* (Sw.) Gill]; *Hydnum abietis* Weir. 300; *H. caput-ursi* Fr. (71748); *H. schiedermayeri* Heuf. (71787-R); [= *H. setosum* Pers.]; *H. septentrionale* Fr. (52037-S) and (72067); *Lentinus Kauffmannii* A. H. Smith 309; *L. lepidus* Fr. 7B; *Lenzites thermophila* Falck. 75A; *Merulius hydnoides* P. Henn. 74; *Omphalia campanella* (Batsch. ex Fr.) Quél. (94075-R); *Phlebia strigosozonata* (Schw.) Lloyd (56454-S); *Pholiota aeruginosa* Peck (1997); *Polyporus albellus* Peck. (14554-S); *P. alboluteus* Ell. & Ev. (72163); *P. fumidiceps* (Atk.) Sacc. & Trott. (59064-S); *P. immitis* Peck (71384); *P. mollis* (Pers. ex Fr.) Fr. 253; *P. paluster* B. & C. 307; *P. radiatus* (Sow. ex Fr.) Fr. (71313-S); *P. resinosus* (Schrad. ex Fr.) Fr. (52043-S); [= *Ganoderma applanatum* var. *laccatum* (Kalchbr.) Rea.]; *P. Schweinitzii* Fr. 102; *P. trabeus* Rostk. (55196-R); *Poria cocos* (Schw.) F. A. Wolf (71693-T); *P. colorea* Overholts & Englerth. (71963-R); *P. ferruginia-fusca* Karst. (1411); *P. luteofibriata* Baxter (2996) and (B); *P. microspora* Overholts 304A; [= *Trametes serialis* Fr.]; *P. nigra* (Berk.) Cooke, 71532-R); *P. sequoiae* Bonar. 306; *Stereum fuscum* (Schrad.) Quél. (71509-Sp); [= *S. bicolor* (Pers. ex Fr.) Fr.]; *S. sulcatum* Burt (71329-R); *Trametes malicola* B. & C. 215 and (71956) and (B); *Trametes sepium* Berk. (71876-S).

Negative by Strip Test.

Boletus luteus Linn. ex Fr. CBS; *B. viscidus* Linn. ex Fr. CBS; *Calvatia saccata* (Vahl.) Morg. CBS; *Clitocybe tabescens* (Scop. ex Fr.) Bres. 203; [= *Armillaria mellea* (Vahl. ex Fr.) Quél. var. *tabescens* (Scop. ex Fr.) Rea]; *Corticium lividum* (Pers. ex Fr.) Fr. (5159) and (15084-R); *Favolus canadensis* Kl. 179 *Fomes applanatus* (Pers.) Gill. (59054-S); [=*Ganoderma applanatum* (Pers.); Pat.]; *F. igniarius* (Linn. ex Fr.) Kicks. 97B; *F. Meliae* (Under.) Murr. (50336-R); *F. pini* var. *abietis* = *F. pini* (Thore ex Fr.) Lloyd, var. *abietis* (Karst) Overholts. (71112); *F. scutellatus* (Schw.) Cooke, 181; *F. tenuis* Karst. (58528-R); *F. tricolor* (Murr.) Sacc. & Trott. 285; *Ganoderma lucidum* (Leyss. ex Fr.) Karst. 89; *G. sp.* (86194); *Hymenochaete corrugata* (Fr.) Lév. (71759-R); *H. rubiginosa* (Dicks.) Lév. (58557-R); *H. tabacina* (Sow. ex Fr.) Lév. (71248-R); *Irpex cinnamomeus* Fr. (67937); *Ithyphallus sp.* (S.); *Polyporus durescens* Overholts. (71096); *P. galactinus* Berk. (52034-S); *P. gilvus* (Schw.) Fr. 133A; *P. glomeratus* Peck, 284; *P. paluster* B. & C. (57010); *Poria corticola* (Fr.) Cooke (71280-R); *P. ferrea* (Pers.) Overholts (B); *Ptychogaster rubescens* Boud. CBS; [= *Ceromyces rubescens* (Boud.) Sacc.]; *Trametes alaskana* Baxter (B); *T. americana* Overholts (18125-R); *T. heteromorpha* (Fr.) Bres. 219; [= *Daedalea heteromorpha* Fr.].

In that Professor Robbins and I have both examined 300 basidiomycetes mostly of the wood-destroying type, it is interesting and useful to compare methods and results. Robbins tested by the streak method from the edge of the growing fungus colony, whereas I tested metabolism solution by the hole method, so that comparison can only be approximate. The species which I describe as "strongly positive" (SP) are those which gave a zone of inhibition which was 50 per cent or more of the expected maximum of 10 mm. If we assume that Robbins' SP species can also be regarded as those which gave a zone of inhibition of 50 per cent or more of his maximum of 55 mm., then out of our respective 300 species we each had approximately 40 SP species. This tends to confirm the impression that, by any method, about 10–15 per cent of the species tested will produce antibacterial substances that merit further investigation.

The comparison becomes more interesting but the agreement less emphasized when those species which were tested by both Robbins and myself are compared. In our respective lists of SP species, mine contains 13 and Robbins' contains 14 species. Table I shows: (A) my list of 13 SP species in order of merit, and (B) Robbins' list of 14 SP species in order of merit. The order of merit is determined by the size of the zone of inhibition produced against *Staph. aureus* only. As my greatest zone width is 9 mm. and Robbins' greatest zone width is 55 mm., I have multiplied my zone size measurements by six to simplify comparison.

TABLE I.—*Comparison of Wilkins' and Robbins' "Strongly Positive" Species.*

(A) Wilkins.		(B) Robbins.	
Zone width (mm. × 6).	Species.	Zone width (mm.).	Species.
54	*Polyporus biformis* (71423-R)	55	*Poria luteofibriata* (B)
48	*Lepiota naucina* (312)	52	*Trametes malicola* (B)
*45	*Daedalea heteromorpha* (59051-R)	*51	*Radulum orbiculare* (57402-S)
	Irpex mollis (71542-R)	*50	*Fomes officinalis* (80518-S)
*39	*Corticium abeuns* (14572-R)	*48	*Polyporus griseus* (14616-R)
*	*Hydnum abietis* (3548)	*45	*Daedalea heteromorpha* (59031-R)
	Polyporus delectans (71089)	*44	*Hydnum abietis* (3548)
*36	*Corticium hydnans* (57407-S)	*43	*Corticium hydnans* (57407-S)
33	*Fomes frazinophilus* (59120-S)		*Fomes ungulatus* (CBS)
30	*Hirneola* sp. (Iowa 42)	40	*Hydnum schiedermayeri* (71787-R)
*	*Fomes officinalis* (80518-S)	36	*Flammula alnicola* (2071)
*	*Pleurotus griseus* (14616-R)	35	*Fomes Meliae* (50336-R)
*	*Radulum orbiculare* (57402-R)		*Trametes sepium* (71876-S)
		*29	*Corticium abeuns* (14572-R)

* Species common to both lists.

In the above lists, 7 species (starred *) are common to both. This indicates some measure of agreement, but it will be noticed that the relative degree of anti-bacterial action, as expressed by size of zone, differs markedly. For example, the three top species in Robbins' list are the three bottom species in the Wilkins' list. Obviously there is even greater discrepancy between those species in either list which do not appear in the other. This can be illustrated by considering only the two top species in each list, as these are representative of all the rest.

	Zone width (mm.).	
	Wilkins.	Robbins.
Polyporus biformis	54	15
Lepiota naucina	48	22

	Zone width (mm.).	
	Robbins.	Wilkins.
Poria luteo-fibriata	55	24
Trametes malicola	52	21

The above shows that 6 of my SP species and 7 of Robbins' SP species are not regarded by the other investigator as in the SP class.

The " streak " method was tested by growing fungi on potato dextrose agar and comparing with the results obtained by Robbins on malt agar and thiamine-peptone agar. The inoculum was a 5 mm. disc. The use of potato dextrose agar was determined by the fact that the bacterial streak is not very successful on malt agar (as mentioned by Robbins), whereas it shows up well on the potato dextrose medium without any coating with another medium. The results obtained by using *Corticium hydnans* are shown in Table II.

TABLE II.—*Comparison of the Streak Method by Wilkins and Robbins.*

	Wilkins.						Robbins.					
	Potato dextrose.			Thiamine-peptone.			Malt.			Thiamine-peptone.		
		Result.			Result.			Result.			Result.	
Time (days).	Diam. (mm.).	*B. coli.*	*S. aureus.*	Diam. (mm.).	*B. coli.*	*S. aureus.*	Diam. (mm.).	*B. coli.*	*S. aureus.*	Diam. (mm.).	*B. coli.*	*S. aureus.*
6	23	10	24	20	21	48	20	2	16	14	12	31
10	40	15	26	36	32	50	36	6	21	20	21	43

The above table shows that, with the same time period, the results differ according to the medium used. Thiamine-peptone appears to be the most favourable for this fungus. It also illustrates the point that there appears to be no direct relation between the size of the colony and the degree of inhibition.

Some of Robbins' results by this method are difficult to interpret. For instance, in the case of *Daedalea heteromorpha* (59051-R) at 6 days the figures are: for *B. coli*, 0, and for *Staph. aureus*, 21 mm.; at 9 days for the same two bacteria the figures are 20 and 11 mm. respectively. That is, in 3 days the *B. coli* figure has increased from 0 to 20 mm., while the *S. aureus* figure has decreased from 20 to 11 mm.

Robbins also tested 44 of the most promising of his fungi by the " disc " method, a modification of the method described by Raper, Alexander and Coghill (1944). By this method he got ' medium ' or ' strong ' positive results on thiamine-peptone or on Czapek-Dox plus cornsteep for the following fungi which appear in my SP list: **Corticium abeuns*, **C. hydnans*, **Hydnum abietis*, *Irpex mollis*, *Lepiota naucina*, **Pleurotus griseus*, *Polyporus biformis*, *P. delectans*, **Radulum orbiculare*.

This confirms previous results as far as the five starred in the above list are concerned. My *Polyporus biformis* and *P. delectans* are also confirmed as SP species by this method. The *Lepiota naucina* of my SP list, Robbins finds relatively inactive on thiamine-peptone, but he got a " medium positive " result for this fungus on Czapek-Dox plus corn-steep medium. The others in my list were presumably not tested by this method. There is, as admitted by Robbins, a certain lack of correlation between the results of the streak method and the disc method but, in the case of those species tested, the disc method of Robbins confirms the SP species as indicated in my original list.

In view of the fact that the thiamine-peptone medium as used by Robbins gave the best results in the streak method, twelve of Robbins' cultures, chosen at random, were grown on liquid malt and on liquid thiamine-peptone. It was found that the results of testing the two metabolism solutions were almost identical. In only one case was the thiamine-peptone result better than that

on malt, and even here the improvement was so slight as to be negligible. The results of this repetition agreed with the previous results as given in my lists, but not with those as determined by the streak method. In view of the fact that by the streak method the thiamine-peptone invariably gave higher results, the above illustrates the point that there is no apparent correlation between zone size in agar medium and that produced by a metabolism solution.

The attention paid to the comparison between the two papers is an attempt to evolve the most satisfactory method(s) for indicating the antibacterial status of any given basidiomycete fungus. I agree with Robbins that " the determination of antibiotic activity by the streak method described above is useful chiefly in eliminating a considerable number of the fungi included in a survey." But even by this method results could be more relatively comparable by attention to the following points :

(1) The time period should be constant.

(2) The medium should be stated.

(3) The medium should be such that it is favourable for the growth of the fungus, and should also support the development of the bacteria.

(4) The inoculation should be made by means of a disc, say 5 mm., as needle inoculations are very erratic in starting, and the time factor is thereby invalidated to some extent.

Under these conditions, the size of the colony is immaterial.

I am not convinced that the disc method of Robbins is an improvement on my strip method, and the latter is regarded merely as an elimination test.

In that, for subsequent chemical isolation and purification of the antibacterial substances, a metabolism solution is required, I am definitely of the opinion that a method which regards the activity of metabolism solution as the criterion of antibacterial action is the most generally useful.

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ELECTRON MICROSCOPE STUDIES OF VIRUS ELEMENTARY BODIES.

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SINCE introduction of the electron microscope a number of different virus elementary bodies have been photographed at high magnification and shown to possess individual characteristics. Thus influenza virus PR8 strain was found to be circular or kidney-shaped in outline, whereas vaccinia was " brick-shaped," and apparently had internal structure with a limiting membrane (Green, Anderson and Smadel, 1942).

Several different elementary bodies have been measured by electron microscopy. Ruska and Kausche (1943) have reported several " brick-shaped " virus elementary bodies to measure as follows : Molluscum contagiosum, 255 × 178 mμ ; ectromelia, 232 × 172 mμ ; myxoma of rabbits, 287 × 233 mμ ; canary pox, 311 × 263 mμ ; and vaccinia, 262 × 209 mμ.

Williams and Wyckoff (1945) reported that influenza A virus measured 75 mμ. Data published by Taylor (1944) revealed that influenza virus A (PR8 strain) measured 101 mμ, influenza virus B (Lee strain) 123 mμ, and swine influenza virus 96·5 mμ, all of which were circular in outline.

Shope papilloma virus was found to be circular in shape with a mean diameter of 44·0 mμ (Sharp, Taylor, Beard and Beard, 1942*a*). Eastern equine encephalomyelitis virus was circular in outline and measured 40·2 mμ (Taylor *et al.*, 1942*b*), and Sharp *et al.* (1942*b*) reported the western variety of the virus to be 40 mμ in size. The size of normal chick embryo component has been ascertained to measure 20 mμ by Taylor *et al.* (1942*a*).

All the above studies have been carried out over a period of years, and simultaneously fresh information has been added to knowledge of the physico-chemical properties of viruses. Recent biochemical studies involving the density measurements of elementary bodies of vaccinia and influenza have suggested that these minute structures are probably surrounded by semi-permeable membranes, through which sucrose may slowly diffuse inwards and water pass outwards, but which are impermeable to bovine albumin. According to Smadel, Pickels and Shedlovsky (1938), and Sharp *et al.* (1944), for a time the density of the virus particle increases, following which the direction of flow changes and the density decreases. Thus it is possible that during the incidental process of centrifugation entailed in the concentration and purification of virus bodies by the usual methods, changes may take place that are liable to affect the size and morphology of the virus particles.

Opinions contrary to these have been expressed by McFarlane, Macfarlane, Amies and Eagles (1939), who concluded that elementary bodies were not bounded by a semi-permeable membrane since they were capable of withstanding drastic treatment such as drying and the action of benzene and ether, which suggested that they were more akin to giant protein molecules than to unicellular structures.

These conflicting views stress the necessity of further investigation into the particulate structure of viruses. The present investigations have been made with these points in mind, and electron micrographs of virus particles have been taken with as little disturbance to tissue as possible, so as to reduce to a minimum the introduction of distortion due to excessive manipulation, such as the grinding of tissues and separation of virus particles from extraneous matter by differential centrifugation.

THE PRESENT INVESTIGATION.

Williams and Wyckoff (1946) have described a method for enhancing the visibility of small particles in electron microscope specimens. The technique consists of placing the virus particles (mounted on a plastic film supported by a fine metal mesh) in a vacuum chamber, and then evaporating metallic chromium atoms from the tip of a hot tungsten filament so that they are deposited obliquely on the virus particle. Thus the metallic atoms, leaving the tip radially, travel in straight lines and form a metallic coating of varying thickness on the specimen so as to accentuate density differences due to minor surface undulations in the object. Thus there will be a piling up of metal on the incident face of the virus particles and a shadow of the particle will be left in the area from which the metal atoms were blocked. The average thickness of the chromium film should be less than 70 Å so that the electrons can penetrate it.

The technique enhances contrast, renders fine structural detail visible, and imparts a three-dimensional effect which makes it easier to visualize the shapes of particles. In addition, the height of the particles may be determined from the shadow-lengths if the angle of incidence of the metal is known, and irregularities in the surface of the particles are shown by corresponding irregularities in the ends of the shadows.

In the viruses studied in this paper, where possible, shadow-cast and non-shadow-cast micrographs have been taken because it will subsequently be shown that different information is provided by the two such pictures. In this manner it is possible to derive the maximum information that the electron microscope is capable of yielding.

A word of caution should be uttered about the interpretation of micrographs of shadow-cast elementary bodies. In the case of particles such as salt crystals, devoid of extraneous debris, the particle height may be obtained directly from the shadow length if the local angle of incidence is known, and for very accurate work, two shadows should be cast in different directions to eliminate errors introduced by sagging of the film. However, in the case of virus particles, there are two other factors to be kept in mind. First, the cap of metal which forms on the incident side of the particle is of such a thickness relative to the virus body that allowance must be made for it when measurements of virus size are made. This is not a serious difficulty, as the thickness of the cap can be calculated if the thickness of the metal film is known. An uncontrollable factor is the difficulty associated with the balance which must be struck, on the one hand,

between the use of specimens containing tissue debris and protein matter adherent to the elementary bodies and, on the other, the risk of introducing artefact and distortion of the elementary bodies by applying drastic treatment necessary to eliminate the surrounding protein. Although unpurified elementary bodies may be masked by a covering film of protein, these disadvantages are compensated for by the advantage of being able to study the natural arrangement of undisturbed masses of elementary bodies. Thus if a sample is shadow-cast in which the viruses are partly buried in protein matter the micrographs may be reasonably sharp, but the shadow-lengths will have little meaning as they are caused only by the portion of the elementary body protruding above the protein film. The surface details, in such a case, will usually be caused by protein macro-molecules adsorbed on the surface of the virus body. All the above factors must be borne in mind if correct deductions are to be drawn from electron micrographs of elementary bodies.

Most of the work in this paper was done with the electron microscope built at Toronto University in 1944; the remainder with an R.C.A. model E.M.U. microscope. The resolution of the R.C.A.* instrument is superior and it is very convenient to use. However, in certain instances it was found that the Toronto instrument was preferable since an objective diaphragm is more readily employed in it to block the widely scattered electrons and thus accentuate the contrast in the final image, which is an important factor in biological specimens where the density differences in the object are small. Both these instruments were calibrated by using a silica replica of a grating, and size measurements agree on the two instruments within the error of the magnification calibration, which is calculated to be approximately 4 per cent.

Materials, Procedures and Results.

This report contains micrographs of three different viruses taken in such a manner as to minimize effects due to distortion of the elementary bodies, and obtain measurements of the particles under conditions as near as possible to those surrounding them when in their natural *milieu*. The presence of elementary bodies in the tissues has been controlled by parallel observation made with the light microscope; thus every specimen examined was known to contain large numbers of elementary bodies.

Molluscum contagiosum.

Method.—A lesion was excised from the skin of a human patient and placed in physiological 0·86 per cent saline. Portions of tissue containing virus were subsequently teased out on glass slides, and after lightly emulsifying them in a small drop of saline the suspension was placed on the usual electron microscope mount consisting of a 10 mμ formvar film supported on a stainless steel mesh. After partial drying, the mounts were well washed with saline and subsequently rinsed with distilled water and allowed to dry. Some of the preparations were examined directly in the electron microscope, and corresponding ones were shadow-cast, as described above, before being examined.

Simultaneously glass microscope slides were prepared of identical material, stained by Paschen's method for virus elementary bodies, and the presence of

* Radio Corporation of America, Camden, N.J., U.S.A.

typical bodies characteristic of the disease was confirmed under the light microscope at × 1000 magnification (van Rooyen, 1938).

Results.—Fig. 1 shows the virus of molluscum contagiosum without shadow-casting. The elementary bodies are free from extraneous debris in the field shown, although in other fields of the same mount they were partially buried in a layer of protein. The bodies may be described as nearly "brick-shaped" and possessing a long and a short axis in all cases. One hundred particles were measured, and the frequency distribution of lengths of the long and short axes are shown graphically in Fig. 2. The median size of the virus may be given as 302 × 226 mμ, with a maximum deviation of 15 per cent.

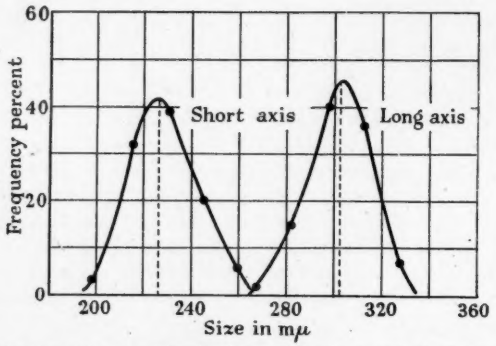


FIG. 2.—These graphs show the size distributions of the long and short axes of one hundred elementary bodies of molluscum contagiosum. Practically all of the deviation from the median values can be accounted for by differences in drying and distortion in the electron image.

Fig. 3 shows the virus again after shadow-casting with chromium at a height to shadow-length ratio of 1 : 5, and the micrograph reveals that the flattening of the bodies is uniform to a surprising degree, and the height is about one-third the length of the virus. Close examination of the picture reveals slight density

DESCRIPTION OF PLATES.

FIG. 1.—Electron micrograph of the elementary bodies of molluscum contagiosum. Note the uniformity of size and shape. × 26,000.

FIG. 3.—Electron micrograph of molluscum contagiosum elementary bodies shadow-cast with chromium, showing minute irregularities on the surfaces of the virus particles. × 13,000.

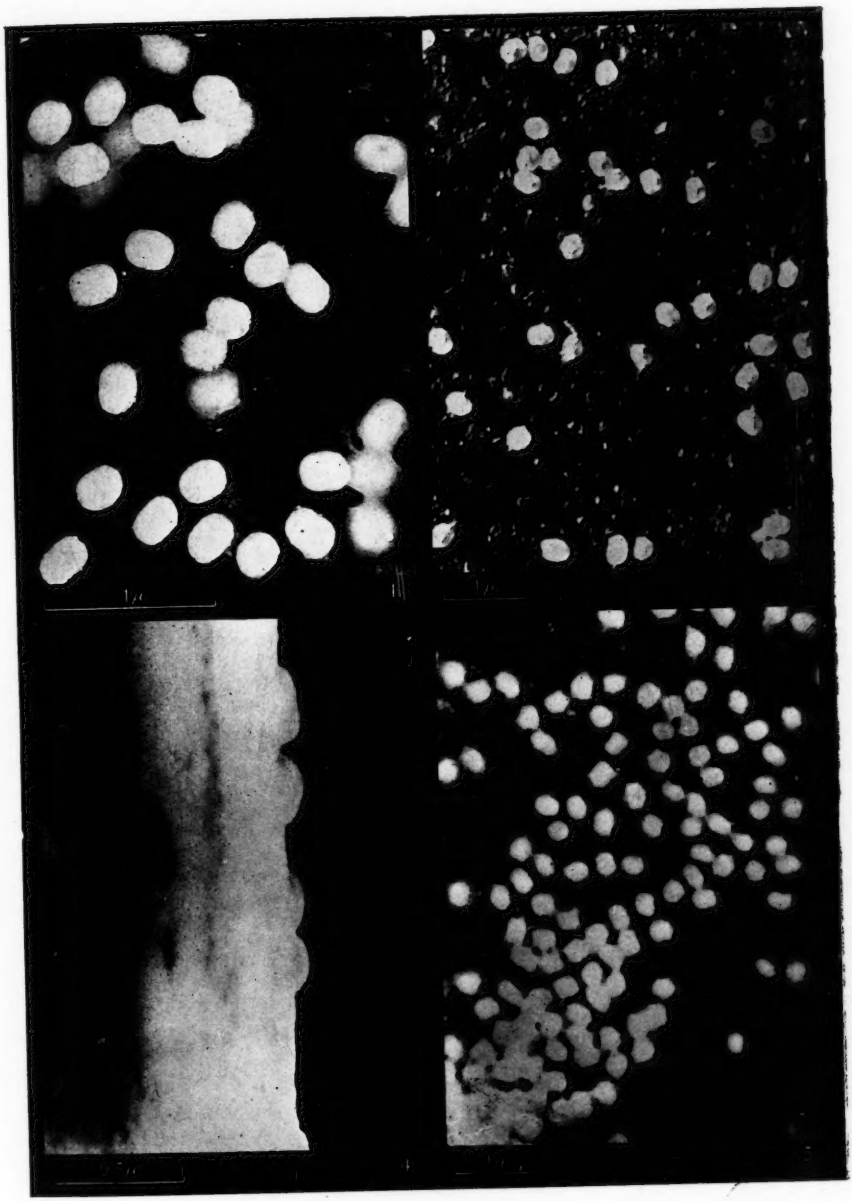
FIG. 4.—Electron micrograph showing a cross-section of molluscum contagiosum virus bodies mounted on a formvar film which has broken and curled back underneath, exposing a side view of the viruses. × 40,000.

FIG. 5.—Non-shadow-cast micrograph of fowl pox elementary bodies. Despite extraneous matter the uniformity in size and "brick-shaped" structure are evident. × 13,000.

FIG. 6.—Electron micrograph of chromium shadow-cast fowl pox elementary bodies, showing uniformity of height and size. The small projections on the surfaces of the virus particles are suggestive of adsorbed protein molecules. × 13,000.

FIG. 7.—Micrograph of an unusual area on a mount of shadow-cast fowl-pox virus. The regularity in spacing and arrangement suggests a quasi-crystalline formation. Irregularities are evident on the surfaces of the particles. × 48,000.

FIG. 9.—Shadow-cast micrograph showing, in the upper part of the field, an ectromelia inclusion body containing elementary bodies embedded in a matrix. The lower part contains a picture of a structure similar in size and shape to an inclusion body, and showing great surface irregularity. × 6,000.



Boswell.

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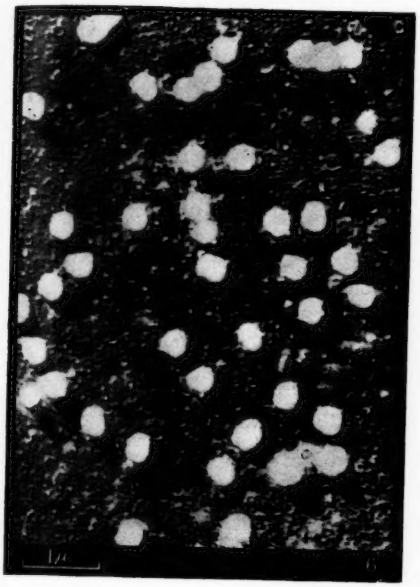

differences in the elementary bodies which are not evident in Fig. 1, and hence are due to the shadow-casting showing up slight elevations and depressions in the surfaces of the virus bodies in the same way that the fine structure in the background has been revealed.

Fig. 4 shows a side view of the virus where the supporting film has curled back; the degree of flattening and relatively smooth appearance are characteristic.

Fowl pox.

Method.—The comb of the cock was scarified and rubbed with a saline emulsion of fowl-pox virus. On the eighth day well-developed vesicles were present, and direct film scrapings revealed numerous fowl-pox-virus particles in stained microscopic slides. At this stage the animal was exsanguinated, the lesions were scraped with the point of a scalpel, and material proved to contain elementary bodies was emulsified in a drop of saline on a glass slide. Electron microscope mounts were prepared as before.

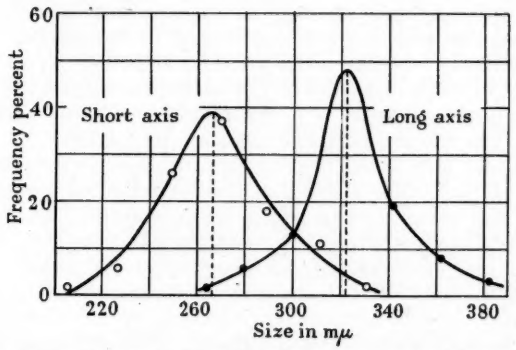

FIG. 8.—Graphs showing the size distributions of the long and short axes of one hundred elementary bodies of fowl pox.

Results.—Fig. 5 shows the virus of fowl pox before shadow-casting. It will be noted that the elementary bodies are partially embedded in some tissue residue, but are nevertheless reasonably distinct, and the "brick-shaped" morphology is clearly indicated.

Fig. 6 shows the virus after shadow-casting; the ratio of object height to shadow-length is about 1 : 5. There is enough protein matter present to make the virus particles appear somewhat oval; the small elevations visible on some of the elementary bodies are thought to be due to protein macro-molecules or small salt crystals. There is a slight indication of minor undulations on the top surfaces of the viruses, but there are no gross irregularities visible, and this is reflected in the fact that the margins of the shadows are smooth. The uniform lengths of the shadows show that the degree of flattening is constant, to a close approximation. Similar to the findings with molluscum contagiosum the heights of the particles are about one-third their lengths.

Fig. 7 shows an unusual area where the elementary bodies were closely packed; there is an interesting appearance of rows and columns and a regularity

of spacing that suggests a quasi-crystalline formation of the virus. It is also evident that the surfaces of the virus bodies are slightly irregular.

One hundred shadow-cast elementary bodies were measured, and the distribution of the lengths of the axes are plotted in Fig. 8. The graphs have the same shape as in the case of molluscum contagiosum but the deviation is greater, and this may be due to protein matter adhering to the elementary bodies, or greater distortion of the bodies on drying than in the case of molluscum contagiosum. The median size of the virus is 322 × 264 mμ, with a maximum deviation of 28 per cent.

Ectromelia virus of mice.

Method.—Twelve mice were inoculated in the feet with a strain of ectromelia virus and five days later six animals showing swelling and oedema of the feet were exsanguinated; scrapings were then prepared from the subcutaneous plantar surfaces of the soles. These were first stained with Paschen's stain and examined under the light microscope for elementary bodies. Positive specimens were selected for the making of electron microscope mounts.

Specimens were also encountered at the stage of the disease where numerous Marchal (1930) inclusion bodies were present, and the opportunity presented itself of being able to study the virus bodies within the membrane in their natural setting. The mounts were made as in the previous cases by suspending some of the exudate from the infected foot in a small drop of saline.

Results.—The top part of the field of Fig. 9 shows part of an inclusion body of infectious ectromelia. The elementary bodies are shown immersed in homogeneous matrix, and the inclusion has a definite outline although there is no positive evidence of a membrane. This mount has been shadow-cast, but due to the associated matter there are not many shadows present. The particles are again "brick-shaped," and their size is estimated to be 300 × 210 mμ, although they could not be measured as accurately as in the previous cases. The degree of flattening is the same as before. A considerable number of these virus inclusions were seen and they ranged in size from 5–10 μ. The lower part of the field shows another structure resembling an inclusion body but containing no virus particles. A number of the latter were observed on the same mounts as those which contained ectromelia virus, and they were generally of the same size range, shape and electron-scattering power as the virus inclusion bodies themselves. It will be observed that there occurs in these objects what may be folds in a membrane or may be long, thin, rod-like structures within a crenated membrane.

DISCUSSION.

The morphology of the three viruses examined was similar, although there were slight size differences. All appeared to be more or less "brick-shaped," although flattened in one direction presumably due to drying; it is believed that in the living state these viruses must occur as "brick-" or "barrel-shaped" entities, and that this appearance is not an artefact due to centrifugation as had been previously suggested. It is still an unsettled point whether the flattening is entirely due to drying or partially due to a spreading out of the virus on the film.

The molluscum contagiosum and fowl-pox viruses showed a size distribution

with a sharp peak and a maximum deviation of only 15 per cent from the median value in the case of molluscum contagiosum and 28 per cent in the case of fowl pox; most of this deviation could be accounted for by slight differences in drying of the elementary bodies and by a certain amount of distortion present in the image. This indicates that the living virus of molluscum contagiosum has a maximum size deviation of $\pm$ 5 per cent from the median value, and that of living fowl pox may have a maximum deviation of $\pm$ 15 per cent from the median. Thus in view of the nature of the distribution it can be stated emphatically that the virus seen in these mounts was not multiplying by any process resembling binary fission or budding.

In the case of ectromelia it is of special interest that several photographs were secured of intact inclusion bodies, containing elementary bodies depicting the size and arrangement of the latter. The same mounts also showed objects identical in appearance with inclusion bodies devoid of virus particles but nevertheless possessing a distinctive structure. The latter are of great interest, and may represent an evolutionary stage in the development of the mature inclusion body. The homogeneous medium in which the elementary bodies occurring in the inclusions are buried is of especial interest, in view of the fact that van Rooyen (1938) has shown with the light microscope and use of the micromanipulator that the inclusion body of molluscum contagiosum contains elementary bodies embedded in a homogeneous, gelatinous, sticky material.

The question of whether or not these viruses possess a cell wall or covering membrane remains unanswered, but some pertinent information has been uncovered. That the virus body is not rigid is indicated by the fact that it flattens out on drying as shown previously; this behaviour is quite different from that of typhoid and coli bacteria, which the author has studied by means of the shadow-casting technique and found to flatten out to a far greater degree than viruses subjected to drying. Further, the constancy of the height of the virus particles is in marked contrast to the great variation in the case of bacteria from one organism to another, which has been attributed to the semi-permeable cell membrane. Another point is that the contents of the bacterial cells clump on drying and the protoplasm forms one or more clumps which shrink away from the cell membrane, and in such occasions the empty cell wall is often exposed. These considerations would indicate that the viruses studied do not have a cell wall akin to that which all bacterial cells studied in the electron microscope have been shown to possess. The continuity of the outline of virus bodies as well as a consideration of their biological activities would naturally lead one to expect the existence of a limiting membrane, but in the case of the viruses reported in this paper no positive evidence has been found to substantiate the existence of a distinct cell membrane.

CONCLUSIONS.

1. Electron micrographs of three animal viruses have been secured, and the metallic shadow-casting technique has been used so as to obtain the maximum of information.

2. Preparations have been made in such a way as to subject the elementary bodies to a minimum of disturbance, with the purpose of ascertaining not only the size and shape of the particles but also their natural arrangement.

3. Only material known to contain elementary bodies as seen in stained

preparations under the light microscope has been selected for electron microscope study.

4. The virus of molluscum contagiosum was found to measure 302 × 226 mμ with a maximum deviation of 15 per cent.

5. The virus of fowl pox was found to measure 332 × 264 mμ with a deviation of 28 per cent.

6. The virus of ectromelia was estimated to measure 300 × 210 mμ although this is not as accurate as in case 4 and 5.

7. In the living state the deviation of virus sizes from the median value must be in each case less than the deviation quoted above, since it is estimated that size variations due to drying and to distortion in the microscope would account for most of the above deviation.

8. In each case the degree of flattening was remarkably uniform, and the height of the particles amounted to approximately one-third of their length.

9. There was no evidence to indicate that the viruses studied showed any signs of multiplication by process of budding or binary fission analogous to that of yeasts or bacteria.

10. Photographs of elementary bodies magnified 100,000 diameters failed to give positive evidence for the presence of a limiting membrane and they appeared to exhibit an undulating surface.

11. The inclusion body of ectromelia has been demonstrated to contain a mass of elementary bodies of nearly uniform size, embedded in a homogeneous matrix.

ACKNOWLEDGMENTS.

Sincere appreciation is expressed to Prof. C. E. van Rooyen for his invaluable direction and encouragement. Prof. E. F. Burton is to be thanked for his kindly interest and co-operation. I am further indebted to Dr. H. A. Dixon, who provided clinical material for investigation, and to Dr. S. G. Ellis for his helpful criticisms and suggestions. The technical quality of the illustrations is due to the interest and ability of Mr. D. R. Welch.

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INTESTINAL BACTERIA AND THEIR PRODUCTION AND CONSUMPTION OF NICOTINAMIDE AS AFFECTED *IN VITRO* BY *p*-AMINOMETHYLBENZENESULPHONAMIDE (AMBAMIDE).

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RECENTLY, Ellinger and Emmanuelowa (1946) reported that orally ingested *p*-aminomethylbenzenesulphonamide (ambamide) changed the composition of the intestinal flora of man in favour of the coliform bacteria and simultaneously increased the urinary elimination of nicotinamide methochloride. The present paper deals with the mechanism of this effect as studied by *in vitro* experiments.

p-Aminomethylbenzene sulphonamidehas been prepared by Miller, Sprague, Kissinger and McBurney (1940) and independently by Klarer (1941). It has been known as mesudin, homosulphanilamide, sulphamylon and marfanil and is now called ambamide in this country. It differs from other sulphonamides in that the amino group is separated from the benzene ring by a methylene group. Compounds with a similar arrangement of the amino group have been prepared and named V 147, V 187 and V 335 (Evans, Fuller and Walker, 1944, 1945; Walker, 1945; Fuller, Tonkin and Walker, 1945). The activity of ambamide against various bacteria has been studied by numerous investigators (Schreus, Brauns and Schümmer, 1941; Domagk, 1942; Link, 1943; Fleming, 1943; Mitchell, Rees and Robinson, 1944; Brownlee, 1944; Brownlee, Dewing, Friedmann, Green and Woodbine, 1945). It is generally agreed that ambamide is more active than any other sulphonamide against anaerobes of the genus *Clostridium*, that *p*-aminobenzoic acid or pus does not interfere with this activity, and that its activity against staphylococci and streptococci equals that of sulphathiazole. No investigations have, however, been published about its action on coliform organisms or other inhabitants of the intestine.

In the present work five main types of experiment have been undertaken:

(1) The effect (*in vitro*) of various concentrations of ambamide on the growth, acid formation, and formation or consumption of nicotinamide† of various strains of *Bact. coli*, *Proteus*, *Shigellae* and intestinal streptococci.

(2) The relation of growth to the formation of acid and nicotinamide by *Bact. coli*, the factors limiting growth, and the effect of nicotinamide on the production of nicotinamide.

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† Nicotinamide in this paper includes nicotinic acid and other compounds indistinguishable from nicotinamide by the microbiological assay with *Lactobacillus arabinosus*.

(3) The effect of formyl ambamide, V 335 and the formyl compound of V 335 on *Bact. coli*.

(4) The nicotinamide content, and the ability to form or consume nicotinamide, of the faecal bacteria of the rat was studied before and during administration of ambamide *in vivo*.

(5) The effect of ambamide (*in vitro*) on the bacterial composition of rat faeces and the production and consumption of nicotinamide.

EXPERIMENTAL.

Material.

The following organisms were used in the course of Experiments 1, 2 and 3. Unless otherwise stated the cultures were obtained from the National Collection of Type Cultures. (*a*) Five strains of *Bact. coli* (1c, 2c and 3c isolated from human faeces, and 2 and 4 from rat faeces). (*b*) *Proteus morgani* 3 isolated from rat faeces. (*c*) *Proteus vulgaris* (No. 401, 3156 and 5887). (*d*) *Shigella sonnei* (No. 2941), *Sh. flexneri* (No. 4832) and *Sh. shigae* (No. 4837).

For Experiments 4 and 5 pellets of faeces were collected in sterile weighing bottles from the ampullae of rats of the Lister stock, or from albinos (from the stock of one of the authors (P. E.)) whose nicotinamide methochloride output was known. About 100 to 150 mg. of the wet faeces was emulsified in 5 ml. peptone water and the remainder dried *in vacuo* to constant weight; from the relation of dry to wet weight the dry weight of the emulsified portion was calculated.

Ambamide, V 335 and the formyl compounds of both drugs were obtained from Messrs. R. F. Reed, Ltd., Barking.

The media used were (*a*) an ammonium-glucose medium (Fildes, Gladstone and Knight, 1933), but with twice the glucose content; (*b*) a casein hydrolysate medium to which vitamins and some amino-acids were added, and which was sterilized by filtration through Seitz filters (Barton-Wright, 1944); (*c*) 20 per cent serum broth.

The rats were maintained from weaning on the mixed diet described by Ellinger (1946).

Methods.

Experiment 1.

Quantities (2·5 ml.) of sterile Barton-Wright medium without and with the addition of 0·02 μg./ml. nicotinic acid, and without and with the addition of ambamide to concentrations from 0·001 to 2·0 mg./ml., were made up to 5 ml. with sterile distilled water. These media were inoculated with thin fresh emulsions from fresh cultures of *Bact. coli* and incubated at 37° for 48 hours. The opacity was then estimated visually, and the acid formed titrated (in aliquot portions) with 0·1 N NaOH using bromothymol blue as internal indicator. The remainder of the culture was used for the estimation of nicotinamide by the method of Barton-Wright (1944). The cultures of *Proteus*, streptococci and *Shigellae* were treated with only one concentration of ambamide (0·02 mg./ml.) in the presence of 0·02 μg./ml. nicotinic acid, but were incubated both aerobically and anaerobically.

All experiments were carried out in duplicate.

Experiment 2.

To obtain information as to whether acid formation can be used as a measure of growth and whether nicotinamide formation is a function of growth, 50 ml. quantities of sterile ammonium-glucose medium pH 6·8 (containing 20 mg./ml. glucose) in 100 ml. conical flasks were inoculated with the various *Bact. coli* strains and incubated at 37° aerobically. Samples of the cultures (5 ml.) were examined at intervals for opacity, acid formation, and nicotinamide content of the supernatant after centrifuging.

To study the influence of pH on growth a similar arrangement was used, the media being brought to the required initial pH by adding 0·1 N HCl. The pH was measured at the beginning and end of the experiment with external indicators. To estimate the influence of nicotinamide and nicotinic acid on growth, acid and nicotinamide formation, the five strains of *Bact. coli* were inoculated into ammonium-glucose medium containing 0·0025 to 0·02μg./ml. either nicotinamide or nicotinic acid ; growth and acid formation were measured after 48 hr. The content of nicotinamide was then examined with *Bact. coli* 3c and 4.

Experiment 3.

To compare the action of formyl ambamide, V 335 and formyl V 355 with that of ambamide, *Bact. coli* 1c was incubated in Barton-Wright medium containing 0, 0·01, 0·02 and 0·04 mg./ml. of the compounds. V 335 was also examined in concentrations of 0·001, 0·002 and 0·005 mg./ml. After 48 hr. growth and acid formation were measured. Groups of two rats (200–250 g.) whose daily urinary nicotinamide methochloride elimination had been examined previously were given either formyl ambamide, V 335 or the formyl compound of V 335 for 6 days at the rate of 2·25 g./day, and the daily urinary elimination of nicotinamide methochloride was estimated by the method of Coulson, Ellinger and Holden (1944).*

Experiment 4.

Rats of both sexes (200–250 g.) were kept in metabolism cages and the daily urinary nicotinamide methochloride elimination was measured as above. The faeces were examined before, during and after the administration of 2·25 g./day of ambamide with the food. Emulsions of the freshly collected faeces were examined for their nicotinamide content immediately after inoculation into, and after aerobic and anaerobic incubation at 37° C. for 48 hr. in Barton-Wright medium containing 0·02 μg./ml. nicotinic acid. The nicotinamide was measured in the medium freed from the organisms by centrifugation.

Experiment 5.

An emulsion of freshly collected faeces in peptone water (25–60 mg. dry weight/ml.) was filtered through a sterile No. 50 filter paper. Serial dilutions (1 : 10) were made from the filtrate in serum-broth with and without the addition of ambamide in concentrations from 2·5 to 100 μg./ml. and incubated for 24 hr.

* Recently KCl available in this country has contained a strongly fluorescent substance which is soluble in isobutanol and interferes with the assay. It can be removed by heating to about 500° C. for 4 hr.

aerobically and anaerobically. After 24 hr. smears were made and examined microscopically.

To obtain some rough information about relative numbers of bacteria in the various dilutions the number of organisms per ml. was estimated by visual assessment of the opacity against a Brown scale. Dilutions in saline were made from some tubes so that about 1000 organisms were contained in 1 ml. medium, and 6 drops of this dilution were inoculated with calibrated Pasteur pipettes on to overdried serum agar plates. These plates were incubated aerobically and anaerobically for about 16 hr., the colonies were counted and smears of the different colonies were examined microscopically. From the second dilution of the control and each ambamide concentration an aliquot portion of the supernatant liquid after centrifugation was assayed for nicotinamide by the method of Barton-Wright (1944). The nicotinamide content of the serum broth was assessed separately and the value deducted from the experimental results.

Results.

Table Ia shows that with *Bact. coli* 1c ambamide in concentrations of 0·002 to 0·02 mg./ml. stimulates growth, acid production and formation of nicotinamide in the organisms. Lower concentrations of ambamide are without any effect and higher ones inhibit all three factors. The presence of nicotinic acid (0·02 mg./ml.) in the medium seems to favour growth and acid production, but not significantly.

TABLE Ia.—*Effect of Ambamide on Growth, Acid Formation and Nicotinamide Production in the presence and absence of added Nicotinic Acid.*

Organism used : *Bact. coli* 1c. medium : Barton-Wright.
Criteria used : (*a*) Relative growth (opacity) assessed in arbitrary units.
(*b*) Acid formed (ml. 0·1 N/ml. culture).
(*c*) Nicotinamide formed (μg./ml.), whole culture.
(*d*) ,, ,, ,, medium only.

Experiment A.				Experiment B.			
Ambamide (mg./ml.).	Criterion.	Nicotinic acid (μg./ml.).		Ambamide (mg./ml.).	Criterion.	Nicotinic acid (μg./ml.).	
		0.	0·02.			0.	0·02.
0	(*a*)	++	+++	0	(*a*)	+++++	+++++
	(*b*)	0·07	0·16		(*b*)	0·24	0·24
	(*c*)	0·055	0·050		(*c*)	0·045	0·050
	(*d*)	0·006	0·004		(*d*)	0·005	0·005
0·001	(*a*)	+++	++++	0·04	(*a*)	+++++	+++++
	(*b*)	0·10	0·17		(*b*)	0·26	0·26
	(*c*)	0·050	0·055		(*c*)	0·053	0·055
	(*d*)	0·004	0·004		(*d*)	0·005	0·005
0·004	(*a*)	++++	++++	0·1	(*a*)	++++	++++
	(*b*)	0·18	0·19		(*b*)	0·22	0·26
	(*c*)	0·085	0·060		(*c*)	0·050	0·053
	(*d*)	0·009	0·005		(*d*)	0·005	0·005
0·02	(*a*)	++++	++++	0·5	(*a*)	++	++++
	(*b*)	0·18	0·18		(*b*)	0·18	0·195
	(*c*)	0·060	0·063		(*c*)	0·035	0·043
	(*d*)	0·006	0·006		(*d*)	0·005	0·005
0·04	(*a*)	+++	++++	2·0	(*a*)	++	+++
	(*b*)	0·10	0·17		(*b*)	0·16	0·16
	(*c*)	0·050	0·063		(*c*)	0·025	0·028
	(*d*)	0·004	0·006		(*d*)	0·006	0·005

Table Ib demonstrates that with *Bact. coli* (strains 2c, 3c, 2 and 4) growth, acid formation and nicotinamide content of the organisms are stimulated significantly by ambamide in concentrations of 0·02 to 0·08 mg./ml.; maximum effects are obtained between 0·02 and 0·04 mg./ml. Very little nicotinamide is released from the organisms into the medium (at least as long as the cells are alive) in the experiments of Tables Ia and Ib.

TABLE Ib.—*Effect of Ambamide on Growth, Acid Formation and Nicotinamide Synthesis by Strains of* Bact. coli.

All experiments in absence of added nicotinamide.
Medium: Barton-Wright.
Criteria used ((*a*) etc.) as in Table Ia.

Ambamide (mg./ml.).	Criterion.	Strain of *Bact. coli*. 2c.	3c.	2.
0	(*a*)	++	++++	++
	(*b*)	0·022	0·156	0·200
	(*c*)	0·008	0·075	0·010
	(*d*)	0·003	0·009	0·005
0·02	(*a*)	+++	+++++	+++
	(*b*)	0·029	0·176	0·330
	(*c*)	0·013	0·083	0·015
	(*d*)	0·005	0·014	0·008
0·04	(*a*)	++++	+++++++	++++
	(*b*)	0·040	0·190	0·400
	(*c*)	0·009	0·090	0·015
	(*d*)	0·005	0·014	0·008
0·08	(*a*)	++++	+++++++	++++
	(*b*)	0·037	0·190	0·330
	(*c*)	0·009	0·079	0·015
	(*d*)	0·005	0·010	0·006

A fourth strain of *Bact. coli* (4) gave results similar to those of 3c.

TABLE Ic.—*Effect of Ambamide on Growth, Acid Formation and Nicotinamide Production by Non-Coliform Intestinal Bacteria.*

The medium used (Barton-Wright, 1944) was supplemented with nicotinic acid (0·02 μg./ml.).
Criteria ((*a*) etc.) as in Table Ia.

Growth conditions.	Ambamide (mg./ml.).	Criterion.	Organism. *Proteus vulgaris* 401.	*Proteus morganii* 3.	*Shigella sonnei* 2941.	*Shigella flexneri* 4832.	*Shigella shigae* 4837.	*Strept. faecalis* 1.
Aerobic	0	(*a*)	+	+	++	+	++	++++
		(*b*)	0·036	0·020	0·088	0·064	0·150	0·330
		(*c*)	−0·020	−0·020	−0·016	−0·017	0	−0·004
	0·02	(*a*)	±	±	++	+	++	±
		(*b*)	0	0	0·090	0·070	0·150	0·020
		(*c*)	−0·020	−0·020	−0·017	−0·016	−0·005	0
Anaerobic	0	(*a*)	+	+	++	+	+	++++
		(*b*)	0·080	0·040	0·170	0·100	0·140	0·270
		(*c*)	−0·020	−0·020	−0·017	−0·016	−0·007	−0·005
	0·02	(*a*)	±	±	++	+	+	±
		(*b*)	0	0	0·170	0·100	0·140	0
		(*c*)	−0·020	−0·020	−0·016	−0·017	−0·013	0

Two other strains of *Proteus vulgaris* (3156 and 5887) gave results similar to those of *Proteus vulgaris* 401.

From Table Ic it can be seen that ambamide (0·02 mg./ml.) (*a*) inhibits under aerobic and anaerobic conditions growth and acid formation of four *Proteus* strains, (*b*) does not affect the three dysentery strains, and (*c*) inhibits growth and acid formation of *Streptococcus faecalis*, the inhibiting effect being greater under anaerobic than aerobic conditions. The nicotinamide consumption by the organisms remains unaffected by ambamide in all cases.

Table IIa shows that with the various strains of *Bact. coli* growth starts at different times (10–16 hr.) after inoculation. For a certain period growth and acid formation go parallel; maximum growth and acid production is reached

TABLE IIa.—*Relation between Growth, Acid Formation and Nicotinamide Production by three Strains of* Bact. coli.

Medium : Ammonium-glucose.
Criteria used : (*a*) Growth (arbitrary units).
(*b*) Acid formed (ml. 0·1 N/ml. culture).
(*c*) Nicotinamide content of medium (μg./ml.).

Time of incubation (hr.).	Criterion.	Strain of *Bact. coli*.				
		3c.		2.	4.	
10	(*a*)	0·2		—	0·1	
	(*b*)	0·04		—	0·020	
	(*c*)	0·010		—	0·010	
14	(*a*)	4·0		—	4	
	(*b*)	0·202		—	0·220	
	(*c*)	0·017		—	0·020	
16	(*a*)	4	4	1	4	4
	(*b*)	0·204	0·204	0·010	0·222	0·220
	(*c*)	0·017	0·017	0·008	0·020	0·021
20	(*a*)		4	3·5		4
	(*b*)		0·226	0·156		0·250
	(*c*)		0·023	0·036		0·036
24	(*a*)		4	4		4
	(*b*)		0·250	0·176		0·245
	(*c*)		0·024	0·036		0·036
40	(*a*)		4	4		4
	(*b*)		0·230	0·178		0·240
	(*c*)		0·030	0·036		0·036
64	(*a*)		4	4		4
	(*b*)		0·220	0·174		0·184
	(*c*)		0·050	0·055		0·095
112	(*a*)		4	4		4
	(*b*)		0·220	0·170		0·184
	(*c*)		0·055	0·073		0·095

Similar results were obtained with *Bact. coli* 1c and 2c.

after 24 hr. The release of nicotinamide into the medium is not significantly affected, but after prolonged incubation (about 40–64 hr.) a sudden considerable rise of the nicotinamide content of the medium occurs simultaneously with a slight increase of opacity and a fall in acid titre, all of which might be due to lysis of the cells. Thus the most suitable time for these observations is after 20–24 hr. incubation.

The effect of pH on the growth of *Bact. coli* is shown in Table IIb. With strains 1c, 2c and 3c growth is optimal at pH 4, but ceases entirely at pH 3·3. With strains 2 and 4 growth is optimal at pH 6·8, gradually diminishes with growing acidity and ceases at pH 3·3.

TABLE IIb.—*Effect of pH on the Growth of Five Strains of* Bact. coli.

Medium : Ammonium-glucose.
Criteria used : (*a*) Growth (arbitrary units).
(*b*) pH at the end of the experiment (48 hr.).

pH.	Criterion.	Strain of *Bact. coli.* 1c.	2c.	3c.	2.	4.
6·8	(*a*)	±	+	+	+++	+++++
	(*b*)	6·4	6·2	6·4	3·9	3·7
6·1	(*a*)	±	++	++	++	++++
	(*b*)	6·1	3·6	3·5	3·8	3·5
5·0	(*a*)	+	++	++	++	+++
	(*b*)	3·5	3·5	3·5	3·8	3·5
4·4	(*a*)	++	++	++	+	++
	(*b*)	3·5	3·5	3·5	3·8	3·5
4·0	(*a*)	+++	+++	+++	+	+
	(*b*)	3·5	3·5	3·5	3·5	3·5
3·3	(*a*)	0	0	0	0	±
	(*b*)	3·3	3·3	3·3	3·3	3·5

TABLE IIc.—*Effect of Nicotinic Acid and Nicotinamide on Growth, Acid Formation and Nicotinamide Production by* Bact. coli (in vitro).

Medium : Ammonium-glucose.
Criteria used ((*a*) etc.) As in Table IIa.

Concentration of nicotinic acid or nicotinamide (μg./ml. medium).		Criterion.	Strain of *Bact. coli.* 3c.	2.	4.
	0	(*a*)	+++	±	++
		(*b*)	0·162	0·033	0·145
		(*c*)	0·053	—	0·087
Nicotinic acid	0·0025	(*a*)	+++	+	++
		(*b*)	0·180	0·038	0·163
		(*c*)	0·070	—	0·105
	0·005	(*a*)	+++	+	+++
		(*b*)	0·183	0·038	0·175
		(*c*)	0·070	—	0·110
	0·01	(*a*)	++++	++	+++
		(*b*)	0·193	0·039	0·188
		(*c*)	0·070	—	0·110
	0·02	(*a*)	++++	+++	+++
		(*b*)	0·198	0·062	0·193
		(*c*)	0·070	—	0·110
Nicotin-amide	0·0025	(*a*)	+++	+	+++
		(*b*)	0·195	0·041	0·165
		(*c*)	0·075	—	0·105
	0·005	(*a*)	++++	+	+++
		(*b*)	0·203	0·043	0·172
		(*c*)	0·075	—	0·120
	0·01	(*a*)	+++++	++	++++
		(*b*)	0·210	0·046	0·186
		(*c*)	0·075	—	0·135
	0·02	(*a*)	+++++	++	++++
		(*b*)	0·213	0·065	0·204
		(*c*)	0·080	—	0·140

The results obtained with ammonium-glucose medium (Table IIc) differed from those with the Barton-Wright medium (Table Ia) in that growth and acid formation by all five coli strains was significantly increased in the presence of nicotinic acid or nicotinamide in concentrations of 0·0025 to 0·02 μg./ml. When the organisms were examined for nicotinamide content (*Bact. coli* 3c and 4) an increased content was found when nicotinamide or nicotinic acid was present in the medium. There is no significant difference between the activity of these two compounds.

Formyl ambamide, V 335 and formyl V 335 (in concentrations of 0·001–0·04 μg./ml.) had no significant stimulating effect on growth and acid production by *Bact. coli* 1c, while V 335 had a definite inhibitory action. Urinary elimination of nicotinamide methochloride was not changed by the ingestion of formyl ambamide, V 335 or its formyl compound.

In rats, ingestion of ambamide with the diet (Table III) caused an increased urinary elimination of nicotinamide methochloride either after a preliminary depression and a latent period (rats 1 and 2), or immediately (rats 3 and 4). When emulsions of the faeces were examined for nicotinamide content, and for nicotinamide synthesis *in vitro*, before, during and after the ingestion of ambamide, the nicotinamide content of the faecal emulsion was in two cases (rats 3 and 4) considerably increased, and in two cases (rats 1 and 2) slightly increased.

TABLE III.—*Effect of Ambamide* in vivo *on the Nicotinamide Content of Faecal Bacteria of Rats and their Production of Nicotinamide.*

Medium: Barton-Wright.

Criteria: (*a*) Urinary elimination of nicotinamide methochloride (μg./day).
(*b*) Nicotinamide content of faecal emulsion (μg./10 mg. dry faeces).
(*c*) Nicotinamide content of medium (μg./10 mg. dry faeces) after aerobic incubation.
(*d*) Nicotinamide content of medium (μg./10 mg. dry faeces) after anaerobic incubation.

2·25 g. ambamide was administered to each rat daily from October 3 to 7.

Experiment.	Criterion.	Date. 1.x.	2.x.	4.x.	5.x.	8.x.	9.x.	11.x.
				Ambamide given.				
1	(*a*)	525	286	338	402	320	1093	660
	(*b*)	0·126	0·101	0·179	0·176	0·103	0·104	0·122
	(*c*)	0·117	0·027	0·028	0·026	0·012	0·005	0·008
	(*d*)	0·127	0·017	0·036	0·016	0·011	0·005	0·009
2	(*a*)	456	372	396	310	469	1202	740
	(*b*)	0·063	0·122	0·167	0·158	0·117	0·145	0·169
	(*c*)	0·011	0·099	0·032	0·032	0·006	0·006	0·006
	(*d*)	0·017	0·038	0·035	0·016	0·005	0·004	0·006
3	(*a*)	154	26·6	51·1	32·6	103	150	64
	(*b*)	0·061	0·089	0·248	0·133	0·154	0·148	0·075
	(*c*)	0·030	0·041	0·099	0·011	0·007	0·012	0·006
	(*d*)	0·025	0·028	0·062	0·007	0·007	0·010	0·006
4	(*a*)	12·3	32·6	58·8	65·4	204	164	64
	(*b*)	0·098	0·069	0·239	0·123	0·293	0·109	0·073
	(*c*)	0·024	0·032	0·024	0·012	0·016	0·018	0·003
	(*d*)	0·028	0·019	0·043	0·015	0·011	0·022	0·004

TABLE IVa.—*The Effect of Ambamide* in vitro *on the Nature of the Faecal Bacterial Flora of the Rat and its Content of Nicotinamide after Aerobic Incubation in Serum Broth.*

(Dry weight of faeces 56 mg.)

Key :
g−, Gram negative; g +, Gram positive; r., rods; c., cocci; d., diplococci; st., streptococci; cb., coccobacilli; sb., spore-bearers.
The number of + signs indicates the relative numbers of the stated types of bacteria: +, 1 in a few fields; ++, 1 or 2 in each field; +++, several in each field; ++++, many in each field.

Ambamide (μg./ml.).	Lowest dilution containing bacteria.	Organisms. Dilution.	Types.	Number/ml.	Plate cultures. Coliforms.	Non-coliforms.	Types of non-coliforms	Nicotinamide content of medium (μg./ml.).
0	10^{-9}	10^{-2}	g − r. +++ g + r. +++ d. ++ st. ++	3000	$1{\cdot}26 \times 10^{8}$	$2{\cdot}40 \times 10^{7}$	cb. small g + c. or d.	2·108
		10^{-7}	g − r. +* d. +	3500	$2{\cdot}25 \times 10^{8}$	0	0	..
		10^{-8}	g − r. +++ d. ++	750	0	$4{\cdot}62 \times 10^{8}$	st.	..
		10^{-9}	d. +++ st. +	50	0	$1{\cdot}68 \times 10^{7}$	large st. g + c.	..
2·5	10^{-7}	10^{-2}	g − r. ++++ d. + st. +	1900	$3{\cdot}10 \times 10^{8}$	$4{\cdot}36 \times 10^{7}$	d.	2·825
		10^{-4}	g − r. + g + c. +++	300	0	$1{\cdot}53 \times 10^{7}$	g + c.	..
		10^{-6}	d. ++ st. ++	500	0	$3{\cdot}22 \times 10^{8}$	g + c.	..
10·0	10^{-6}	10^{-2}	g − r. ++++ d. + st. +	3000	$1{\cdot}16 \times 10^{9}$	0	0	5·625
		10^{-4}	d. ++	2300	$1{\cdot}36 \times 10^{9}$	$5{\cdot}90 \times 10^{5}$	st.	..
		10^{-6}	d. ++ st. ++	400	0	$1{\cdot}32 \times 10^{9}$	st.	..
40·0	10^{-6}	10^{-2}	g − r. ++++ g + r. sb. ++ d. +	2000	$2{\cdot}10 \times 10^{9}$	$2{\cdot}00 \times 10^{7}$	g + c.	4·250
		10^{-4}	g − r. +++ g + r. sb. ++ d. +++ g + c. +++	1300	$1{\cdot}68 \times 10^{9}$	$7{\cdot}80 \times 10^{6}$	g + c.	..
		10^{-6}	st. +	50	0	$2{\cdot}63 \times 10^{7}$	d., st.	..

* Bad smear.

Two similar experiments with ambamide concentrations between 10–100 μg./ml. gave similar results.

TABLE IVb.—*The Effect of Ambamide* in vitro *on the Nature of the Faecal Bacterial Flora of the Rat and its Content of Nicotinamide after Anaerobic Incubation in Serum Broth.*

(Dry weight of faeces 56 mg.)

Key as in Table IVa.

Ambamide (μg./ml.).	Lowest dilution containing bacteria.	Organisms. Dilution.	Types.	Number/ml.	Plate cultures. Coliforms.	Non-coliforms.	Types of non-coliform.	Nicotinamide content of medium (μg./ml.).
0	10^{-15}	10^{-2}	g − r. +++ g + r. ++ d. ++ long st. ++++	2000	$2{\cdot}8 \times 10^{7}$	$3{\cdot}02 \times 10^{8}$	d.	2·000
		10^{-4}	g − r. ++ d. ++ long st. ++	750	0	$1{\cdot}06 \times 10^{8}$	g + c. d.	..
		10^{-6}	g − r. + d. ++ st. +	900	0	$1{\cdot}24 \times 10^{9}$	d. large g + c.	..
		10^{-15}	d. ++ st. ++ g + c. +++	900	0	$1{\cdot}71 \times 10^{9}$	d. large g + c.	..
2·5	10^{-6}	10^{-2}	g − r. +++ d. +++ st. +++	1600	$1{\cdot}24 \times 10^{8}$	$3{\cdot}52 \times 10^{7}$	g + c.	2·625
		10^{-4}	g − r. + d. ++ st. ++	1100	$1{\cdot}87 \times 10^{7}$	$5{\cdot}50 \times 10^{8}$	d.	..
		10^{-6}	g + c. ++ d. ++ st. ++	600	0	$5{\cdot}06 \times 10^{8}$	d. st.	..
10·0	10^{-12}	10^{-2}	g − r. ++ g + r. + long st. +++	1100	$2{\cdot}16 \times 10^{8}$	$7{\cdot}37 \times 10^{7}$	g + c.	5·250
		10^{-4}	g − r. +++	800	0	$1{\cdot}25 \times 10^{8}$	g + c.	..
		10^{-6}	g − r. + d. ++ st. ++	800	0	$6{\cdot}42 \times 10^{8}$	d.	..
		10^{-12}	d. + st. +	400	0	$3{\cdot}45 \times 10^{8}$	d. st.	..
40·0	10^{-6}	10^{-2}	g − r. +++ d. +	1000	$1{\cdot}23 \times 10^{9}$	$4{\cdot}20 \times 10^{7}$	d.	4·000
		10^{-4}	g − r. ++ d. +	800	$6{\cdot}32 \times 10^{8}$	$7{\cdot}06 \times 10^{7}$	d.	..
		10^{-6}	d. +	50	$2{\cdot}25 \times 10^{8}$	$8{\cdot}20 \times 10^{6}$	d.	..

Two similar experiments with ambamide concentration between 10–100 μg./ml. gave similar results.

Tables IV*a* and IV*b* show that in serum broth cultures of faecal bacteria, the coliform flora disappears and the non-coliform flora increases in number with increasing dilution. This occurs both in aerobic and anaerobic cultures. Ambamide increases significantly the number of coliform bacteria in the more concentrated emulsions as well as the synthesis of nicotinamide in both aerobic and anaerobic cultures. Maxima are reached for growth of coliforms at ambamide concentrations of 40 μg./ml. in this experiment and of 10 μg./ml. in another. Nicotinamide production was maximal at 10 μg./ml. ambamide in all experiments. Ambamide causes a most striking change in the qualitative composition of the faecal flora, particularly after aerobic incubation.

DISCUSSION.

The synthesis of nicotinamide by a number of intestinal bacteria in pure culture *in vitro* has been shown by Burkholder and McVeigh (1942) and Thomson (1942). The investigations of Ellinger and Emmanuelowa (1946) made it probable that the contribution of the intestinal flora to the nicotinamide requirements of the human body (Ellinger and Coulson, 1944; Ellinger, Coulson and Benesch, 1944; Ellinger, Benesch and Kay, 1945; Najjar, Holt, Johns, Medairy and Fleischmann, 1946) was mainly due to the production and release of the compound by coliform bacteria. *p*-Aminomethylbenzenesulphonamide proved to be a valuable tool in these investigations. It is evident (Tables Ia, Ib) that it increases the growth, acid production and nicotinamide synthesis of various strains of *Bact. coli* in pure culture and of the mixed cultures from rat faeces (Tables IV*a*, IV*b*). In these mixed cultures ambamide does not only stimulate the growth of coliform bacteria, but inhibits the growth of non-coliform organisms, some of which (such as *Proteus* and streptococci) consume nicotinamide (Table Ic). In pure culture these latter bacteria are inhibited by ambamide (Table Ic). Nicotinamide formation by *Bact. coli* in pure and mixed culture seems to go parallel to a certain degree with growth, but higher ambamide concentrations seem to retard nicotinamide synthesis (Tables Ia, IV*a*, IV*b*). An acidity of pH 3·3 stops growth and nicotinamide formation (Table IIb).

When still viable, the *Bact. coli* strains examined do not release into the medium much of the nicotinamide produced, but there is a considerable release when lysis occurs (Table IIa). This is of importance from the point of view of the role played by the intestinal flora *in vivo*. It was not possible to obtain detailed information about the proportion of dead bacteria in the various parts of the intestine; but there is no doubt that a large portion of the bacteria are dead in the distal region of the gut (Cruickshank and Cruickshank, 1931), and nicotinamide is readily absorbed from these regions (Ellinger and Hardwick, 1947).

The greatest stimulating effect of ambamide on *Bact. coli* was obtained in pure and mixed cultures with concentrations of 4 to 40 μg./ml. (Tables Ia, Ib, IV*a*, IV*b*), while concentrations above 100 μg./ml. showed a definite inhibitory effect (Table Ia). *Proteus* strains and *Streptococcus faecalis* (Table Ic) were completely inhibited, however, by ambamide concentrations of 20 μg./ml.; this is in agreement with Brownlee's (1944) findings on streptococcus P 90 and *Staphylococcus aureus* 133. The stimulating effect on *Bact. coli* and the inhibitory effect on non-coliforms did not differ significantly under aerobic and anaerobic conditions,

Two similar experiments with ambamide concentration between 10–100 μg./ml. gave similar results.

although in mixed cultures the stimulating effect on coliforms was somewhat more pronounced aerobically (Tables Ic, IV*a*, IV*b*).

The presence of nicotinamide and nicotinic acid (0·0025–0·01 μg./ml.) in the medium favoured equally growth, acid production and nicotinamide synthesis; higher concentrations had no greater effect (Table IIc). This explains the fact that in Barton-Wright medium (which contains a certain amount of nicotinamide) additional nicotinamide did not show any effect (Table Ia).

It was found necessary to sterilize the Barton-Wright media by filtration through Seitz filters since autoclaving produced a rise in the apparent nicotinamide content by the microbiological assay method with *Lactobacillus arabinosus*. Each successive autoclaving raised the apparent nicotinamide content still further. It was not possible to ascertain whether this effect was due to an increase of nicotinamide or nicotinic acid since the amounts involved were too small to be identified by a chemical method; the microbiological assay gave unmeasurable values for the unautoclaved medium and values of 0·003 μg./ml. and 0·005 μg./ml. after the first and second autoclavings. Formation of nicotinamide or nicotinic acid on heating the medium might be due to a reaction similar to that obtained by Bovarnick (1943*a*, *b*, *c*; 1944) on heating asparagine and glutamic acid in the presence of manganese salts; our medium, however, was free from asparagine. Bovarnick (1944) believed that she had obtained chemical proof that nicotinamide is formed, but the evidence for the identification of nicotinamide in the ether extracts of the heated compounds is not conclusive, since we were unable to extract nicotinamide in measurable amounts from pure aqueous solutions of nicotinamide; this substance seems to be practically insoluble in ether.

In the *in vivo* experiment on rats (Table III), ambamide produced an immediate rise in nicotinamide methochloride elimination in two cases, and after a latent period in the other two even after discontinuation of ambamide. This is in agreement with the findings on rats and on one human subject by Ellinger and Emmanuelowa (1946). The cause of this delayed action of ambamide (which usually acts without delay in human beings) is not known, but might be found in the concentration of ambamide in the intestines. The nicotinamide content of the faecal bacteria was significantly increased in all rats examined when ambamide was ingested, and continued to increase for a short while even after discontinuation of ambamide; it fell to the original level after four days. There was, however, no increased release into the medium when the faecal emulsion was incubated (Table III).

The observations reported in this paper explain the findings of Ellinger and Emmanuelowa (1946) that administration of ambamide to human beings increases the coliform flora of the faeces and the urinary output of nicotinamide methochloride.

SUMMARY.

1. Ambamide (4–40 μg./ml.) stimulates the *in vitro* growth, acid production and nicotinamide synthesis of coliform bacteria in pure and mixed culture. It inhibits the growth of non-coliform bacteria, such as *Proteus* or *Streptococcus faecalis*, which consume nicotinamide. Higher concentrations of ambamide have an inhibiting effect on coliform bacteria.

2. Growth, acid production and nicotinamide synthesis by *Bact. coli* go

parallel to a certain extent: they are inhibited by acidity of more than pH 3·5. The presence of small amounts of nicotinamide or nicotinic acid in the medium favours growth, acid production and nicotinamide synthesis.

3. Intact cells of *Bact. coli* release little nicotinamide into the medium, but much more is released after lysis.

4. In rats ambamide increases the nicotinamide content of the faecal flora and the urinary elimination of nicotinamide methochloride.

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THE ASCORBIC ACID CONTENT OF THE HYPERTROPHIED HUMAN PROSTATE.

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WE have not found in the literature any statements about the amount of ascorbic acid in the human prostate. The relations of the prostate, and of ascorbic acid, to steroid metabolism makes this matter worth investigation.

All amounts of ascorbic acid are stated as μg. per g. of fresh tissue.

METHODS.

We have not had the opportunity of examining the normal human prostate in a condition sufficiently fresh for reliable analyses of ascorbic acid. Human prostates, removed on account of benign hypertrophy, were brought as quickly as possible from the operating theatre to the laboratory; those from other hospitals were conveyed in a vacuum flask on ice. Prostates removed too late in the day for the completion of the estimations were kept frozen overnight; comparative analyses recorded below showed that no serious loss occurred under these conditions. Some prostates can be kept frozen for 3 days without loss (see Table I, Case 14). The prostate was weighed, thawed if necessary, and cut up; after selection of pieces for histological examination portions of about 1 g. were weighed for analysis. These were cut up with scissors and ground with sand in 3 per cent metaphosphoric acid in a mortar; a mechanical disintegrator was inefficient owing to the extreme toughness of the material. Maceration in HCl (50 c.c. conc. Analar acid in 100 c.c.) was tried, but had no consistent effect either upon disintegration or upon the yield of ascorbic acid. The tissue, when ground, was mixed and centrifuged with three successive portions of 10 c.c. of 3 per cent metaphosphoric acid, and the fluid titrated with 2:6 dichlorophenol-indophenol. Portions examined by the hydrazine method of Roe and Kuether (1943) were ground without sand in trichloracetic acid.

The agreement, usually within 10 per cent, between estimations upon different portions of the same prostate was as close as one would expect in view of the varying histological appearance of such portions.

RESULTS.

The results are shown in Table I and Fig. 1.

Twenty-six prostates were examined, of which 22 were from patients who had not received ascorbic acid as such. The weights of the organs ranged from 7

to 115 g. ; where no ascorbic acid had been given the results of the great majority (42 out of 56) of the estimations lay between 50 and 110 μg. Altogether exceptional figures were (1) those for Case 16 (25 μg.)—this prostate was the smallest of the series (7 g.) and was very dense ; (2) a single amount of 146 μg. in Case 1 ; and (3) a discordant result in Case 10. All the prostates showed the histological characters of benign senile hyperplasia. No definite correlation could be found between histological appearances, which were, indeed, very similar

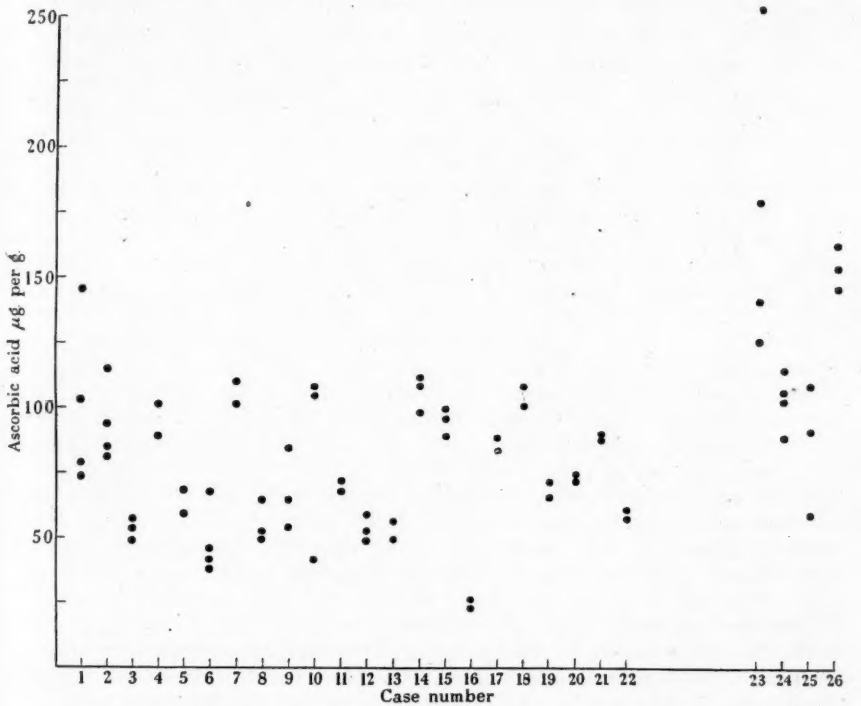

FIG. 1.

in almost all the specimens, and the concentration of ascorbic acid, except in the very fibrotic Case 16 referred to above. If one compares sections of the two smallest prostates, namely, No. 16 (weight 7 g., ascorbic acid 25) and No. 20 (weight 8 g., ascorbic acid 75), certainly the former shows larger areas of fibrous and muscular tissue without epithelium. But such comparisons are not very conclusive, owing to the difficulty of sampling. A small carcinoma (area in section about 7 × 7 mm.) was found by Dr. G. J. Cunningham elsewhere in prostate 24, but the amount of ascorbic acid was not exceptional. Of the four cases (No. 23 to 26) who had received from 2·1 to 5·2 g. ascorbic acid before the operation, two showed in some areas higher concentrations (252, 178, 162) than did any of the others.

TABLE I.

Case number.	Weight of prostate. (g.)	Ascorbic acid μg. per g. fresh weight. 2:6-dichlorophenolindophenol.				Hydrazine.	Ascorbic acid given before operation.
1	17	103	79	74	146	—	—
2	60	115	85	94	82	—	—
3	50	57	49	54·5	—	—	—
4	22	101	89·1	—	—	—	—
5	19	59·1	67·9	—	—	—	—
6	115	47·6	37·4	68·5	43·5	—	—
7	68	101·6	110·7	—	—	—	—
8	66	49	64	—	—	53	—
9	14	84	65	—	—	61·5	—
10	34	107	42·4	104·5	—	—	—
11	15	71·2	67	—	—	—	—
12	38	52·2	57·8	48·4	—	—	—
13	110	56·5	49·2	—	—	—	—
14	29	111·7	97·9	108 (3 days)	—	—	—
15	62	89·7	96·7	—	—	99	—
16	7	25·6	23·9	—	—	—	—
17	44	87	83·4	—	—	—	—
18	58	107	100	—	—	—	—
19	32	71·6	65	—	—	—	—
20	8	72·6	74·5	—	—	—	—
21	43	87·1	87·9	—	—	—	—
22	60	60·3	57·5	—	—	—	—
23	35	252	178	140	125	—	2·6 g. in 13 days
24	42	106	103	114	87, 82	—	2·1 g. in 7 ,,
25	42	92	58·5	108	108	—	5·2 g. in 26 ,,
26	36	162	145	—	—	153	3·2 g. in 8 ,,

Ascorbic acid in human autopsy material.

The data obtained by Jaworsky, Almaden and King (1934) within 24 hours of death, by 2:6-dichlorophenolindophenol titration, from 67 autopsies are those which usually appear in the literature to show the concentration of ascorbic acid in human organs. The figures show a very wide range of variation, from 3 times to 1/10 of the average. "Approximately 20 per cent of the cases gave evidence of a condition of latent scurvy." The amounts decreased with advancing age. For comparison with our data for the prostate one may take their age group of 46–77 years (Table II).

TABLE II.—*Ascorbic Acid in Human Autopsy Material. Age-group* 46–77. *Means of* 19 *Cases* (*Jaworsky, Almaden and King*).

Adrenal	230	Kidney	47
Brain	110	Lung	45
Pancreas	95	Heart	21
Liver	64	Thymus	46
Spleen	81		

Loeper, Cottet and Lesure (1936) in 10 human livers at autopsy found concentrations ranging from 44 to 250, with two amounts of 316 and 1390.

These figures thus show the same 30-fold range as do those of Jaworsky *et al.* The significance of averages drawn from such material, which probably represent a mixture of the effects of fatal disease and post-mortem change, is doubtful.

DISCUSSION.

Giroud (1938) summarized the literature relating to the distribution of ascorbic acid in the organs of man and animals. The only data for the prostate which he records, presumably from his own analyses, were obtained from the bull (200) and stallion (110), but nothing is said of the number of animals examined.

The only quantitative estimations of ascorbic acid in any fresh human organ, apart from the eye, which we have found in the literature are those of Policard and Ferrand (1936*a* and *b*), who examined 8 ovaries and 14 corpora lutea in cases requiring gynaecological operation. The method depended upon titration with 2:6-dichlorophenolindophenol (Manceau, Policard and Ferrand, 1936). The analyses were begun a quarter of an hour after the excision. The amounts ranged from 110 to 380 in the ovary, and from 230 to 1030 in the corpus luteum, where the concentration rises with the growth of the organ after ovulation. The ovaries may not have been normal, but, as in the case of the prostate, human material which is certainly normal is not obtainable.

The range of concentration (almost all between 45 and 110) found in these prostates covers that of the averages given by Jaworsky *et al.* (Table II) for the human organs, brain, pancreas, liver, spleen, lung and thymus, at autopsy, and reaches the lowest figure (110) found by Policard and Ferrand in the fresh ovary. The liver of mice of the low cancer strain C57 (Kennaway, Kennaway and Warren, 1944), or of Wistar rats, has about twice this concentration of ascorbic acid, and we have found amounts of 90 to 110 in the livers of healthy guinea-pigs on the ordinary laboratory diet. The hyperplastic prostate consists of a matrix of fibrous tissue and plain muscle, containing alveoli lined by epithelium, of which several types can be distinguished. The epithelial cells do not make up a very large fraction of the whole bulk of tissue, so far as one can judge from the appearance of sections; the organ contrasts in this respect with an almost wholly epithelial organ like the liver. Hence if the ascorbic acid in two such organs as the hypertrophic human prostate and the liver of the guinea-pig is about the same in total amount, and lies chiefly in the epithelium, the concentration in these cells in the prostate must be high. Dr. G. J. Cunningham is examining the histological distribution in the hypertrophic prostate of the reduction of silver nitrate in a strongly acid medium which is stated to show the presence of ascorbic acid.

SUMMARY.

Hypertrophic human prostates contain ascorbic acid in concentrations usually between 50 and 110 μg. per g. In the literature only one other series of estimations of ascorbic acid in a fresh human organ (the ovary) has been found.

We wish to express our thanks to Prof. Geoffrey Hadfield for accommodation in his department; to Dr. G. J. Cunningham for the histological examination of the material; and to the British Empire Cancer Campaign for a grant which

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INFECTIOUS ECTROMELIA: EXPERIMENTS ON INTERFERENCE AND IMMUNIZATION.

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IN 1936, Greenwood, Hill, Topley and Wilson reported that they could immunize mice against the virus of infectious ectromelia by the following procedure: A dose was first given of infective liver filtrate fully inactivated by treatment with 0·2 per cent formalin for 10 days in the cold. A second dose was given 7 days later of virus similarly treated with formalin, but only for 3 days; this still contained living virus as judged by tests in normal mice, but produced no infection in mice previously injected with "wholly dead" vaccine. After the two doses, first of inactive, second of "attenuated" virus, mice withstood a big dose of living virus. Greenwood *et al.* made no detailed study of the mechanism of this immunizing procedure, which was used as a tool in their studies of experimental epidemiology. Similar findings are recorded for the virus of African horse sickness (Du Toit and Alexander, 1930); here also immunization against living virus can be achieved by injecting horses first with tissue suspensions wholly inactivated with 1 : 1000 formalin, later with virus incompletely killed by treatment with weaker formalin; 1 : 2000, 1 : 3000 and 1 : 4000 formalin were used for treating the virus used for the successive doses. It seemed to us unlikely that incomplete formalinization would affect virus particles uniformly, attenuating them all without killing them. Recent work on the interference phenomenon in the virus field suggested that the results might be better explained on the basis that a partly inactivated vaccine contained a mixture of live and dead virus, the dead component of the mixture interfering with the full activity of the live virus. We accordingly carried out experiments similar to those of Greenwood *et al.*, but in order to avoid the complications introduced by the presence of formaldehyde or urotropin we used

virus inactivated with ultra-violet light. We were in any case on surer ground in so doing, for virus inactivated by ultra-violet light has been shown to interfere with live virus in the case of bacterial viruses (Delbrück and Luria, 1942) and influenza (Henle and Henle, 1944).

Methods: (a) *Inoculation of mice.*

Earlier work was carried out with the Hampstead strain of ectromelia described by Marchal (1930). Later we used also a Moscow strain kindly given us by Prof. V. Soloviev. The two were antigenically alike. Mice injected intraperitoneally with either strain developed diverse pathological lesions, varying with dosage and other factors. After a heavy dose most mice died within a few days with extensive liver necrosis. Others, more resistant, or after a smaller dose, died later with general peritonitis associated with fat necrosis due to pancreatic damage. Yet others developed generalized skin-lesions involving feet, tail, mouth and nose, as described by Marchal; some of these recovered. Though we took full precautions against spread of infection from one cage to another, the Hampstead strain of virus proved to be much less infectious than was described by Marchal; even within a cage cross-infection occurred irregularly. The Moscow strain was, however, highly infectious. On inoculation into the pad the Hampstead virus produced good local lesions: fatal general infection might or might not follow. The Moscow virus, on the other hand, usually killed mice after pad-infection; with higher dilutions mice commonly died with hepatic necrosis without showing any local foot-lesion.

For virus filtrates to be used in the studies to be described, we usually infected 9 mice intranasally with potent virus, killed them after 3 or 4 days and removed the lungs. Lungs of any dead mice in the group were pooled with these, and a $2\frac{1}{2}$ per cent suspension made in buffered saline (0·85 per cent NaCl + M/60 Sörensen phosphate buffer pH 7·4). After centrifugation the suspensions were clarified either through paper-pulp and sand filters or through coarse (A.P.D. 2μ and 1μ) gradocol membranes and then filtered through 0·7μ membranes. Suspensions made thus from lung material proved much clearer and easier to filter than those made from liver, and gave filtrates of higher titre. Broth, which helps virus-filtration so much, was not used in these experiments, in order to avoid adding substances which might interfere with ultra-violet inactivation.

In all experiments, unless otherwise stated, surviving mice were killed and autopsied after 21 days.

(b) *Ultra-violet light inactivation.*

The apparatus employed in these experiments for the controlled exposure of infectious fluids to ultra-violet light is shown diagrammatically in Fig. 1 (*a*) and (*b*).

The liquid flows at a known rate through a capillary tube of transparent quartz (1·5 mm. bore; 1 mm. wall), a known length of which is exposed to the light issuing from the slot in the case surrounding a $\cap$ type low-pressure mercury discharge lamp. The length of capillary exposed can be varied by altering the effective length of the slot by means of the sliding cover—the maximum length is 10 cm. The light emitted by this type of burner (supplied by the Thermal Syndicate, Ltd.) consists to the extent of 95 per cent of $\lambda = 2536$ Å, the intensity at 100 cm. horizontally from the lamp being $1{\cdot}2 \times 10^3$ ergs/cm.^{2}/sec. The

quartz capillary is in the same horizontal plane as the ∩ tube and situated 2·5 cm. from one arm. Rates of liquid flow have varied from 0·25 to 2·5 c.c./min., with actual exposure varying from 1·25 sec. up to 125 sec. The exposures greater than 50 sec. were attained by using a transparent quartz coil made of tubing of the same bore and wall thickness as the straight capillary.

Two variables enabled the rate of flow to be adjusted, (1) the glass capillary tube *R* of 0·5 mm. bore and length which varied from 5 to 25 cm., and (2) the value of *h*, the mean height of liquid producing flow. The glass and quartz tubes, after thorough cleaning in hot chromate-sulphuric acid mixture, were sterilized in the hot air oven and then assembled as in Fig. 1 (*b*) with sterilized

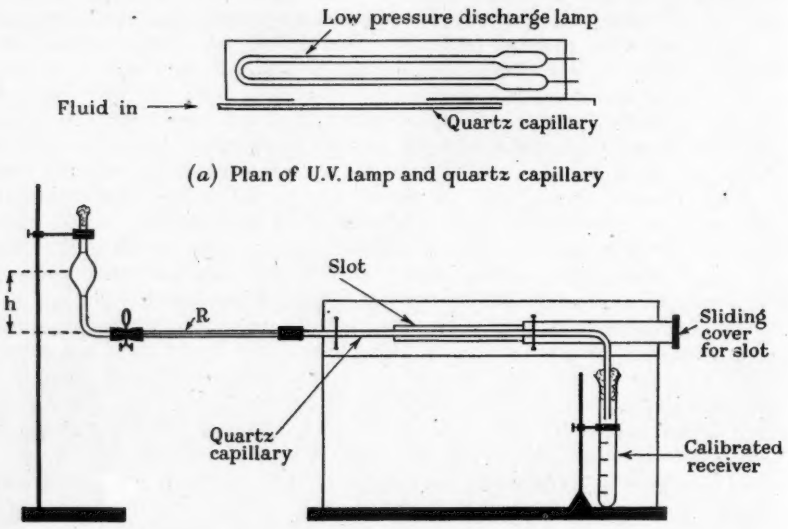

FIG. 1.—Apparatus for controlled exposure of infectious liquids to ultra-violet light.

rubber unions. In operation, it is important to stop the flow of liquid when the meniscus reaches the lower graduation of the bulb, for should it be inadvertently allowed to pass the resistance *R* the rate of flow of the final portion of liquid will be much increased and the whole experiment will be vitiated.

The discharge tube generates some heat, and the fluid passing at the lowest rates of flow became slightly warmed; starting at 20° C. the liquid in the receiver would be 25° C. Strictly, therefore, we were investigating the combined effect of ultra-violet and heat radiation.

Results: Amount of irradiation necessary to inactivate.

The top line of Table I shows that a filtrate irradiated for 2 seconds was not completely inactivated, while one irradiated for 4 seconds apparently was; also that both vaccines had good immunizing power. Two doses of 0·5 ml. were

given intraperitoneally one week apart. After another week a challenge dose of about 200 MLD was given, also intraperitoneally. The results are shown in the lower part of Table I. The filtrate from which the vaccine was made had a moderately high titre, killing 2 of 3 mice specifically when diluted 10^{-5}.

TABLE I.—*Effect of Time of Irradiation on Activity and Antigenicity.*

	Controls (unvaccinated).	UVR 1 sec.	2 sec.	4 sec.
Challenge	††††††	†††ESS	†SSSSS	SSSSSS
	††††††	..	SSSSS	†SSSSS

† = specific ectromelia death.
E = generalized ectromelia.
S = survived 3 weeks.

In a similar experiment with virus of possibly higher titre (10^{-5} killed 4/5 mice, 10^{-6} 1/5) irradiation for 4 sec. did not wholly inactivate, since 1 of 6 injected mice died. Treatment for 16 sec. was, however, effective; no mice died and virus could not be recovered on passage. In a number of later experiments 16 sec. irradiation never failed to render all filtrates non-infectious.

Immunizing power of ultra-violet-ray-treated filtrates.

As already seen in Table I, two intraperitoneal doses of vaccine protected against a heavy dose of virus given subsequently by the same route. This result was confirmed in all subsequent experiments. Even when the titre of the filtrate was as low as 10^{-4}, vaccine made by irradiation for 16 sec. was effective. The second dose of this vaccine was not necessary, a single intraperitoneal dose giving good immunity against a challenge 14 days later. Irradiation for 25, 50, or even 65 sec. still yielded an active vaccine; but after 105 and 125 sec. antigenic power was apparently lost.

Activity of these vaccines, made from the Hampstead strain of virus, was only certainly demonstrable when both vaccine and challenge-dose were given by the intraperitoneal route. Intraperitoneal vaccination failed to protect against the development of foot lesions, nor did subcutaneous vaccination prevent deaths after intraperitoneal challenge. Limitation to the intraperitoneal route for both injections was not, however, so necessary in work with the Russian strain to be described in a later paragraph.

Interference phenomenon.

In a mixture of live and inactive virus given intraperitoneally, the activity of the live virus was wholly or largely suppressed.

Table II shows the results of titrating a virus intraperitoneally using as diluent (*a*) buffered saline, (*b*) undiluted 4 sec. vaccine, (*c*) undiluted 16 sec. vaccine.

All mice of the lots surviving an injection of virus-vaccine mixture were challenged at the end of 3 weeks with a dose of virus which killed 6/6 controls: all 34 of them survived.

TABLE II.—*Interference Phenomenon (Intraperitoneal Injections).*

Virus diluted in	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
(*a*) Control (buffer)	†††††*	††††SS	†**SS	†††††*	††††SS
(*b*) In 4 sec. vaccine	†SSSS	†SSSSS	†ESSSS	SSSSSS	SSSSSS
(*c*) In 16 sec. vaccine	..	..	*SSSSS	†ESSSS	..

† = specific death.
E = generalized ectromelia.
S = survived 21 days.
* = non-specific death.

It will be seen that in the presence of 4 sec. or 16 sec. vaccine, a very definite sparing effect is evident against the effects of live virus.

Similar results were obtained in subsequent experiments.

Nature of the interfering agent.

All evidence suggested that the interfering agent consisted of inactivated particles of virus. A potent 16 sec. vaccine lost all interfering activity after filtration through a gradocol membrane (A.P.D. 0·2μ) which was known to retain all virus. Further, after centrifugation at 8000 r.p.m. for 1 hour in an Ecco centrifuge, the supernatant fluid was found to be devoid of interfering activity, while the deposit, resuspended in the original volume of fluid, was still active.

Treatment with ultra-violet rays for longer periods removed the interfering property roughly *pari passu* with antigenicity. After 25 and 50 sec. of irradiation activity was still present, while after 65 sec., and in one test after 50 sec., activity was lost. These results do not differ greatly from those reported earlier for the effect of ultra-violet rays on antigenic activity. Henle and Henle (1947) found that in the case of influenza viruses, antigenicity persisted longer after irradiation than did interfering activity.

Interference was only demonstrable if the filtrate before irradiation had a titre of about 10^{-4} or better. When this was so, a vaccine had a definite delaying effect on deaths of mice even when mixed with an equal volume of living undiluted virus filtrate. A reduction of mortality was seen when the proportions of live : inactive virus were 1 : 9 or more (Table II). If less vaccine was used, the effect was not demonstrable. When, for instance, we took a mixture of 16 sec. vaccine and 1 in 100 live virus, this only killed 1 of 6 mice ; but a 1 : 10 and a 1 : 100 dilution of this mixture killed, in each instance, 5 of 6 mice ; the intraperitoneal inoculation in all cases was 0·5 ml. Such a result recalls the familiar reactivation by dilution of an apparently neutral virus-antiserum mixture—the " dilution phenomenon." The mechanism may, however, be quite different in the present instance.

Some suppression of viral activity was seen when vaccine was injected intraperitoneally 18, 24, 48 or 72 hours before active virus, or along with it. When virus preceded vaccine by 18, 24, 48 or 72 hours no such suppression occurred (Table III).

TABLE III.—*Intraperitoneal Injection of Vaccine Before Virus and vice versa.*

	18 hours.	24 hours.	48 hours.	72 hours.	Mixture.
Vaccine before virus 1 : 100	††SSS .	††ESSS .	ESSSSS .	†SSSSS .	*†SSSS
Virus 1 : 100 before vaccine	†††††† .	†††††E .	†††††† .	†††††† .	..

Virus 1 : 100 killed 6/6 mice at the beginning of the experiment and 5/6 after keeping 5 days in the cold.

Other routes of injection.

The Hampstead strain of ectromelia was irregularly lethal in our hands when given intracerebrally, subcutaneously or intravenously; we were, however, able to study the effect of virus-vaccine mixtures given intranasally or into the foot-pad. We were unable to demonstrate any consistent interfering effect when we used the intranasal route. When we injected into the foot-pad mixtures which were harmless when given intraperitoneally, local foot-lesions were produced as readily as if no inactive virus had been present. Strangely enough, however, there were no deaths in the groups receiving the mixtures (Table IV). An experiment with the Moscow strain gave a similar result.

TABLE IV.—*Interference Phenomenon (Intraplantar Injections).*

	Dilutions in buffered saline.		Dilutions in vaccine.	
	V/10^3.	V/10^4.	V/10^3.	V/10^4.
1st exp. . .	††††FF	†††FFF .	FFFFFF	FFFFF0
2nd exp. . .	††††††	FFFFFF .	FFFFFF	FFFFFF
	FFFFFF	000000 .	FFFFFF	FF0000

† = specific death.
F = local foot lesion.
0 = no lesion.

Mixture with irradiated (16 sec.) virus produced no evident effect on foot-lesions on intraplantar injection, but none of 12 mice so treated died, while 7 of 12 controls did so. When vaccine was injected into pads 24 hours before 1 : 10,000 virus, only 1 of 12 mice died, and in addition foot-lesions were less severe.

Experiments with the Moscow strain.

The necessity, with the Hampstead strain, of giving both vaccine and challenge dose by the same route, the intraperitoneal, raised the question of whether the phenomenon was one of immunity in the ordinary sense at all. The resistance induced within 24 hours was presumably a manifestation of "interference"; could not that found after a fortnight be equally due to a persistence of the same effect, rather than to true immunity? The question was not easily settled in experiments with the Hampstead strain of ectromelia, but the more invasive, more infectious Moscow virus enabled us to show that irradiated vaccine could immunize against virus introduced at another site, and hence that local interference could not by itself explain our findings.

The Moscow virus proved to be inactivated by the same dosage of ultra-violet-radiation as was effective for the other strain. Two intraperitoneal doses of 0·5 ml. 16 sec. vaccine a week apart induced some immunity against intra-plantar injection of living virus and against susceptibility to contact infection. For the last test, mice were exposed in the same cage with newly infected mice (intraperitoneal or pad) 7 days after their second vaccine dose, and left for 3 weeks.

The vaccination failed to protect completely against local foot lesions, though their severity was modified. In this and in two other experiments there was definite reduction of mortality (Table V). In another experiment all 6 vaccinated

TABLE V.—*Immunization with Moscow Strain.*

	Mice receiving 2 doses 16 sec. vaccine i.p.	Control mice.
Intraperitoneal challenge V 1 : 100	SSSSSS	††††††
Intraplantar challenge V 1 : 100	FFFFFF FFFFF0	†††††† ††††FF
Intraplantar challenge V 1 : 1000	FFFFFF FFFFFF	†††††† †††FFF

† = specific death.
F = foot lesion.
0 = no lesion.

mice survived contact exposure to infected mice during 23 days; within this period 5/6 similarly exposed controls died of ectromelia. It seems then that the resistance found 14 days after vaccination probably represents true immunity.

Must effective vaccines contain living virus?

We have shown that a little live virus may be masked by an excess of inactive virus; this raises the question of whether some so-called killed vaccines may not contain masked living virus, and even of whether a little living virus may not be essential for their activity. In hopes of settling this question we inoculated mice intraperitoneally with mixtures of vaccine and live virus in the proportion of 100 : 1, 1000 : 1, and 10,000 : 1. The virus used turned out to be active only to a dilution of 10^{-3}, so the mice receiving the 10,000 : 1 mixture were not further considered. Three mice of each of the other two groups were killed after 3, 7 and 14 days, and suspensions of their livers passed intraperitoneally to more mice to test for the presence of virus. From the 100 : 1 mixture virus was recovered after 3 and 7 but not 14 days; lung suspensions, tested after 14 days, also proved negative. No virus was obtained from livers of the 1000 : 1 group after 3, 7 or 14 days. It is possible, therefore, for the live virus to be masked so thoroughly that serial passage fails to reveal it; but in the experiment recorded this was so only when minimal quantities of live virus were used.

Another line of approach indicated that effective vaccines need not contain living virus. As already described, we could not demonstrate any local interfering effect when we gave mixtures of vaccines and minimal amounts of virus

into the foot-pad. We did obtain vaccines which effectively immunized but yet produced no lesions on pad-inoculation. We have therefore no reasons for doubting that ectromelia virus completely inactivated by ultra-violet rays may still be able to immunize.

DISCUSSION.

The experiments described indicate that ectromelia virus inactivated by ultra-violet radiation will immunize mice against living virus, and that this immunizing action need not depend on the presence in the vaccine of any living virus. The latter conclusion was not easily reached, for inactivated virus was found capable of interfering with or masking the activity of considerable quantities of live virus mixed with it. Though the conclusion as regards ectromelia is that a little living virus in a vaccine is not necessary for its activity, our results call in question the validity of some published work on durable immunity after killed vaccine injections. Casals and Olitsky (1945), for instance, found lifelong immunity in mice after injection of irradiated virus of Russian spring-summer encephalitis. Before such a result is accepted, one would like to know whether the possibility has been excluded that a little living virus has been masked by an excess of inactivated virus. Again, when inactivated vaccines are found to be unexpectedly labile, the possibility should be considered that the lability may be due to the dying off of a small but necessary fraction of masked living virus. Though we have not carried out parallel experiments with formolized virus, our results could provide a rational basis for the vaccination methods used by Greenwood *et al.* (1936) in ectromelia and by Du Toit and Alexander (1930) in horse sickness. Experiments now in progress show that living vaccinia virus can " interfere with " the lethal results of ectromelia infection in mice. It remains to be shown whether the same fundamental mechanism is necessarily concerned in all examples of virus " interference."

SUMMARY.

Ectromelia virus inactivated by ultra-violet irradiation can interfere with or mask the activity of living virus mixed with it. It is not necessary to conclude that irradiated ectromelia vaccines only immunize because of a masked living virus component. The findings do, however, call for examination of the possibility that masked living virus may be present in other inactivated virus vaccines and may even play a part in their efficacy.

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THE EFFECT OF EXPERIMENTAL INCREASES IN THE γ-GLOBULIN AND ALBUMIN CONTENT OF SERA ON THE RESPONSE GIVEN BY TURBIDITY AND FLOCCULATION TESTS.

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EXPERIENCE has shown that the various serum turbidity and flocculation reactions used in the clinical laboratory respond differently with sera from patients with different diseases. It was hoped originally that one or other of these tests would be specific for liver disease, but it has since been shown that positive reactions are produced primarily when the concentration of some particular protein fraction is greater than in normal serum. In liver disease it appears to be principally the γ-globulins which show such an increase; any reagent, therefore, which reacts mainly with γ-globulins will give a positive reaction with the majority of sera from liver cases, and also with other sera in which this protein fraction is increased. Other tests give positive reactions in sera in which the lipoid-bearing protein fraction is abnormally high (Recant, Chargaff and Hanger, 1945). But such preferential response is always relative, and it may be assumed that none of these empirical tests gives a specific reaction with any protein fraction. Comparative tests are therefore not only useful but necessary.

Several methods may be used to investigate the efficiency of the various tests. Firstly, large numbers of results may be examined and classified into various categories. Secondly, the product formed between the reagent and the serum proteins may be analyzed. Thus Maclagan (1944) has investigated the chemical composition of the precipitate formed in his Thymol test, and Wunderly and Wuhrmann (1946) have demonstrated (by electrophoretic measurements) the extent of precipitation of the various protein fractions by cadmium sulphate. Finally, studies may be made of the reaction between the test reagent and isolated serum protein fractions: Kabat, Hanger, Moore and Landow (1943) have followed the reaction between various concentrations of γ-globulin and two of the reagents (cephalin-cholesterol and colloidal gold). The γ-globulin was obtained by electrophoretic fractionation from normal sera and from sera from cases of hepatitis and cirrhosis. These authors were able to establish the important fact that all the samples of γ-globulin gave the same response with these two reagents. Illness is often followed by an increase of γ-globulin, but it appears that, chemically, the latter is always the same whatever the disease.

In the present work we have made a comparison of the Thymol-turbidity

test (Maclagan, 1944), the Cephalin-cholesterol-flocculation test (Hanger, 1938), the Takata reaction (Jezler, 1934) and the Cadmium-turbidity test (Wunderly and Wuhrmann, 1945). Only the last test employs a reagent which is a simple salt solution; the reagents in the other tests are more or less complex colloidal systems. It is well known that in such systems there is a tendency towards reduction of the surface area of the colloidal dispersed phase. As a result the degree of dispersion does not remain constant, but decreases to an extent which also depends on temperature. This instability makes it very difficult to define precisely the reactivity (with serum) of such colloidal reagents. It was in order to obtain a stable reagen tgiving reproducible results over long periods of time that we developed the Cadmium test. In this test all the factors governing the state of the reagent are well defined and its behaviour with serum is constant. Since, however, the cadmium reagent is of molecular dispersion we foresaw that it might have a different sensitivity from the other (colloidal) reagents. A comparison of various factors involved in the four tests is set out in Table I. It will be seen that dilution of the serum is smallest in the Cadmium test, which

TABLE I.—*Comparison of Different Factors in the Four Turbidity Tests.*

Test.	Dispersion of reagent.	Dilution of serum.	pH.	Time before reading result.
Thymol-turbidity	Partly colloidal	1 : 60	7·8	1 hr.
Cephalin-cholesterol-flocculation	,, ,,	1 : 25	7·0	24 ,,
Takata-reaction	,, ,,	1 : 2 up to 1 : 128	9·4	24 ,,
Cadmium-turbidity	Molecular	1 : 1·5	7·0	5 min.

is therefore closest to *in vivo* conditions. Any dilution of serum changes the solubility of the proteins and tends to decrease their stability; this is because some disaggregation of protein complexes takes place, a fact which has been demonstrated experimentally by Elford, Graber and Fischer (1936) by ultrafiltration studies. The Thymol and Takata tests (especially the latter) are carried out in distinctly alkaline conditions. This has a stabilizing effect, as at this pH (well above their iso-electric point) the serum proteins are in the form of complex anions, i.e. carrying a strong net negative charge. With the Cadmium test the result is obtained in a very short time (5 min.), whilst the Cephalin and Takata tests require 24 hr. In general the reading of tests in which colloidal components are involved should not be delayed, since complex processes such as " ageing " and " ripening " are apt to take place and it is difficult to obtain comparable results. In the Thymol and Cephalin tests the solution of the reagent is on the verge of supersaturation, and both are therefore primarily sensitive to any factor likely to change their solubility. In the Takata test the alkaline colloidal solution of HgO is similarly sensitive. The turbidity produced with serum in the Cadmium test is due to dehydration of the protein molecules. According to the lyotropic series of Hofmeister the effect of the anion SO_4'' is stronger than that of the cation Cd^{++}. As the salt, $CdSO_4$, is very soluble in water and the reaction takes place in almost undiluted serum, the question of solubility is of no importance.

Consideration of the four factors enumerated in Table I shows that the four serum turbidity reactions are essentially empirical, and that only experiment can determine their relative merit.

EXPERIMENTAL.

Methods.

Tests employing colloidal reagents.

The procedures for the Thymol turbidity and Cephalin-cholesterol flocculation tests have been given by Maclagan (1944), Kunkel and Hoagland (1946), and Shank and Hoagland (1946), and by Hanger (1938) respectively. That for the Takata reaction has been described recently by Schneiderbauer (1946) and by Klima and Bodart (1944). The reading of the Thymol turbidity test was carried out with a Zeiss Stufenphotometer fitted with special appliances for the estimation of relative as well as absolute intensities. The light filter used was L_2. Turbidities of 20 units and under were recorded as $-$; 20 to 30 as $\pm$; 30 to 50 as $+$; 50 to 100 as $++$; and 100 and above as $+++$.

Cadmium test.

The detailed technique was as follows: To the serum (0·4 ml.) in a dry tube was added 0·4 per cent $CdSO_4$, $8H_2O$ (0·2 ml.) and the tube shaken gently. (The reagent was added with a special dropping pipette standardized (see 'Pharmacopoe Helv. V') so that 20 drops equalled 1 ml. distilled water at 15° C.; 4 drops reagent were added to each tube of serum.) The test was read after 5 min., and if the original serum was itself slightly turbid comparison was made with this. Relative turbidities were assessed visually and assigned arbitrary values ($\pm$ to $+++$). The highest value was given only if a strong and permanent turbidity was produced with less than 4 drops of reagent. This simple method of assessment has been preferred in order to make the method practicable for routine clinical work.

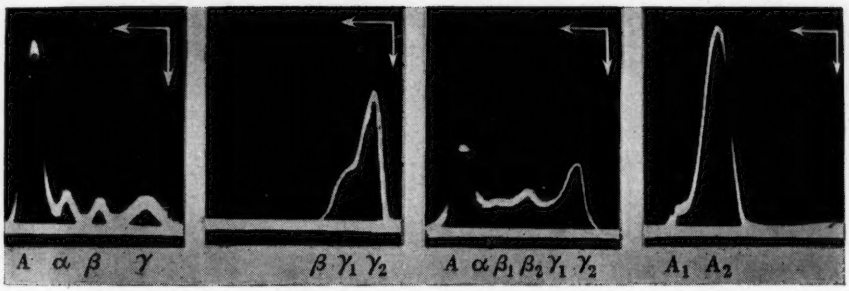
Serum protein fractions.

γ-Globulin.—This was prepared from normal human serum. Saturated $(NH_4)_2SO_4$ solution was added to bring the final concentration of this salt to 33 per cent, and the precipitated γ-globulin separated. After redissolving it was again precipitated, and the whole process then repeated once again. The final solution of the protein was dialysed 24 hr. against running tap water and for 8 hr. against distilled water. Enough saline was then added to obtain a 3 per cent solution of the γ-globulin. The material was checked by electrophoretic analysis (Table II and Fig. 1) and the protein found to contain 97·9 per cent γ-globulin. The degree of purity attained (further details are given by Wunderly, 1947) equals that of Kekwick (1940), who used a method in which the γ-globulin was thrice precipitated with Na_2SO_4.

Albumin.—A preparation of normal human serum-albumin ("salt-poor") from the Cutter Laboratories (Berkeley, California, U.S.A.) was used. Electrophoresis showed that it was practically pure albumin (Table II and Fig. 1). There was only a small component X, amounting to 4·2 relative per cent, which had a higher mobility than the albumin. Since the preparation was described as "salt-poor" it is improbable that this gradient was caused by salt; neither is it an ε gradient. It may possibly be due to the slight denaturation which sometimes occurs as a result of purification procedures (Wiedemann, 1947).

TABLE II.—*Protein Composition.*

Protein.	Fraction.	Relative percentage.	Absolute percentage.	Mobility (μ).	Time (sec.).
Serum (normal)	A	70·5	5·1	8·14	8400
	α	3·9	0·3	5·90	
	β	11·2	0·8	4·43	
	γ	14·4	1·1	1·81	
γ-Globulin	β	2·1	0·1	4·23	8400
	γ_1	29·0	1·8	3·05	
	γ_2	68·9	4·2	1·84	
Serum T (hepatitis)	A	36·3	2·2	7·55	9300
	α	9·1	0·5	5·75	
	β_1	6·8	0·4	4·74	
	β_2	13·6	0·8	3·81	
	γ_1	5·2	0·4	2·67	
	γ_2	29·0	1·8	1·40	
Albumin	X	4·2	0·3	11·35	7200
	A	95·8	7·2	9·24	



Serum (normal). γ-Globulin. Serum T (hepatitis). Albumin.

FIG. 1.—Electrophoretic Patterns of Serum and Serum Fractions.

Electrophoretic measurements.

Electrophoresis was carried out by the conventional procedures (Tiselius-Philpot-Svensson) in an apparatus constructed by Strübin & Co. (Basle). The patterns illustrated in Fig. 1 show descending boundaries with reduced basis line (Wiedemann, 1946). The potential gradient was 3·78 volts per cm., the temperature 2° C., and the pH 7·9. The buffer system used was sodium veronal/sodium acetate/hydrochloric acid (Michaelis). The mobility (μ) was measured in cm.$^3 \times 10^5$/sec. $\times$ volt; ionic strength was $\mu = 0{\cdot}1$.

Plan of Experiments.

Before applying the four flocculation and precipitation tests to pathological sera, twelve normal sera were first examined. One of these, in which all four tests were negative, was chosen for the first two main experiments. In the first experiment (Table III) seven tubes were set up containing decreasing amounts of normal serum and increasing amounts of γ-globulin. In the next experiment (Table IV) the six tubes contained a constant amount of normal serum and γ-globulin but increasing amounts of albumin.

TABLE III.—*Variation of Serum Content and γ-Globulin.*

Tube no.	Serum (normal) (ml.).	Globulin (3 per cent solution) (ml.).	Protein composition (relative percentage). Albumin.	Globulins. α.	β.	γ.	Total protein (per cent).	Results of test. Cadmium.	Cephalin.	Thymol.	Takata.
1	0·80	—	70·5	3·9	11·2	14·4	7·30	−	−	−	−
2	0·75	0·05	68·4	3·8	10·9	16·9	7·04	±	+	−	−
3	0·70	0·10	66·5	3·7	10·6	19·2	6·82	±	++	±	−
4	0·65	0·15	64·0	3·6	10·3	22·1	6·51	++	+++	±	−
5	0·60	0·20	61·9	3·5	10·0	24·6	6·25	+++	+++	+	−
6	0·55	0·25	60·6	3·4	9·7	26·3	5·86	+++	+++	++	−
7	0·50	0·30	59·2	3·3	9·4	28·1	5·47	+++	+++	+++	−

TABLE IV.—*Variation of Albumin; Serum and γ-Globulin Constant.*

Tube no.	Serum (normal) (ml.).	γ-Globulin (3 per cent solution) (ml.).	Albumin (25 per cent solution) (ml.).	Saline (0·85 per cent) (ml.).	Protein composition (absolute percentage). Albumin.	Globulins. α.	β.	γ.	Total protein (per cent).	Results of test. Cadmium.	Thymol.	Cephalin.
8	0·5	0·3	0·10	0·30	4·25	0·11	0·34	1·18	5·86	++	+++	+++
9	0·5	0·3	0·15	0·25	5·27	0·11	0·34	1·18	6·90	+	++	+++
10	0·5	0·3	0·20	0·20	6·31	0·11	0·34	1·18	7·94	±	+	++
11	0·5	0·3	0·25	0·15	7·35	0·11	0·34	1·18	8·98	−	±	+
12	0·5	0·3	0·30	0·10	8·39	0·11	0·34	1·18	10·02	−	±	+
13	0·5	0·3	0·35	0·05	9·44	0·11	0·34	1·18	11·06	−	−	

TABLE V.—*Variation of Serum Content (Hepatitis) and Albumin.*

Tube no.	Serum T (hepatitis) (ml.).	Albumin (5 per cent solution) (ml.).	Protein composition. Albumin.	Globulins. α.	β1.	β2.	γ1.	γ2.	Total protein (per cent).	Albumin globulin.	Results of test. Cadmium.	Cephalin.	Thymol.	Takata.
14	1·0	—	36·0	9·1	6·8	13·6	5·2	29·0	6·10	0·57	+++	+++	+++	+++
15	0·9	0·1	42·0	8·2	6·2	12·4	4·8	26·4	5·99	0·68	+++	+++	+++	++
16	0·8	0·2	47·7	7·3	5·6	11·2	4·4	23·8	5·88	0·85	+++	+++	+++	+
17	0·7	0·3	53·1	6·5	5·0	10·0	4·0	21·4	5·77	1·10	+++	+++	+++	±
18	0·6	0·4	58·8	5·7	4·4	8·8	3·5	18·8	5·66	1·40	++	+++	++	−
19	0·5	0·5	64·4	4·9	3·8	7·6	3·1	16·2	5·55	1·82	++	+++	++	−
20	0·4	0·6	70·1	4·1	3·2	6·4	2·6	13·6	5·44	2·47	+	+++	++	−
21	0·3	0·7	75·8	3·3	2·6	5·2	2·1	11·0	5·33	3·51	±	++	+	−
22	0·2	0·8	81·5	2·5	2·0	4·0	1·6	8·4	5·22	5·64	±	++	+	−
23	0·1	0·9	87·1	1·7	1·4	2·8	1·2	5·8	5·11	12·00	±	+	+	−
24	—	1·0	100·0	—	—	—	—	—	5·00	—	−	−	−	−

TABLE VII.—*Variation of Serum Content (Hepatitis).*

Tube no.	Serum T (hepatitis) (ml.).	Saline (0·85 per cent) (ml.).	Protein composition (mg. per cent). Albumin.	Globulins. α.	β1.	β2.	γ1.	γ2.	Total protein (mg. per cent).	Results of test. Cadmium.	Cephalin.	Thymol.	Takata.
25	0·60	0·20	1648	416	311	635	238	1327	4575	+++ inst.	+++	+++	+++
26	0·40	0·40	1099	277	207	423	159	883	3048	+++ inst.	+++	+++	+++
27	0·20	0·60	550	139	103	212	79	441	1524	+++ inst.	+++	++	++
28	0·10	0·70	275	69	52	106	39	221	762	+++ inst.	+++	++	++
29	0·05	0·75	138	34	26	53	20	110	381	++	+++	+	±
30	0·02	0·78	56	14	10	22	8	42	152	+	++	+	−
31	0·01	0·79	28	7	5	11	4	21	76	−	+	±	−

In order to estimate the effect of the presence of a high concentration of lipoid-carrying globulins, serum (T) from a case of hepatitis was used in the third experiment. The eleven tubes in this series of tests (Table V) contained decreasing amounts of serum T and increasing amounts of albumin. The protein distribution in these tests was particularly interesting as the hepatitis-serum T contained only 36·3 rel. per cent albumin, but no less than 20·4 rel. per cent lipoid-carrying β-globulin (Table II and Fig. 1). It is thus in striking contrast to normal human serum. As would be expected, every flocculation test with serum T was strongly positive.

The history of the case from which serum T was obtained was as follows: A woman (T—), aged 69, showed on admission to hospital typical signs of an infectious hepatitis of three weeks' duration. There was intense jaundice coloration of the skin and mucosa, with sudden onset of enlarged liver, spleen and lymph-nodes; slight fever at the onset; urine bilirubin, ++. The blood picture is shown in Table VI. During the period of clinical observation the usual therapeutic measures were taken (vitamins B, C and K, suprarenal hormone, diet and confinement to bed). The general state improved and the liver and spleen showed a tendency to return to normal proportions.

TABLE VI.—*Laboratory Tests with Hepatitis Serum T.*

Date.	Bilirubin content (Heymans v. d. Bergh) (mg. per cent).	Sedimentation rate (mm.).	Prothrombin time (Quick) (per cent).	Weltmann Band (per cent).
29.x.46	30·0	16–28	80	0·250
8.xi.46	22·7	25–39	—	0·175
21.xi.46*	12·7	27–47	90	0·175
3.xii.46	5·5	17–31	90	0·175
17.xii.46	5·3	38–49	90	0·100
6.i.47	2·7	50–57	70	0·150

* Sample used for electrophoretic analysis (see Fig. 1).

Our findings with this serum compare well with the results obtained by Martin (1946) in his electrophoretic study of the components of serum protein in infective hepatitis.

Results and Discussion.

A general survey of the results obtained in these experiments with the four tests shows that the relative sensitivity of the Cephalin, Thymol and Cadmium tests to experimental changes in protein composition of serum is remarkably constant. On the other hand the Takata reaction gives a less marked response. It has been pointed out that the nature of the reagents used is very different, and it would not have been surprising if a greater difference in their relative sensitivities had been found. The results given in Table III show that the Cephalin, Thymol and Cadmium tests respond almost equally to an increase of γ-globulin in normal serum. The critical concentration of γ-globulin (at which positive reactions begin to be obtained) is *ca.* 17–19 rel. per cent, the albumin concentration still being 66–68 rel. per cent. The results given in Table IV (influence of variation of albumin content) show that the Cadmium test gives a negative result at a lower albumin concentration than the Cephalin or Thymol tests; the latter are still positive at an albumin value of 83 rel. per cent, a value which is never attained *in vivo*. The results of the Takata test were negative throughout and are not tabulated.

The effect of increase of the albumin content of hepatitis serum (Table V) is most marked with the Takata test. The results given by the other three were almost identical. It follows that an increase of the lipoid-carrying α and β globulin fractions is of considerable importance. If increased, their influence on the response given by the tests is less easily overcome by an increase in albumin than is the effect of increased γ-globulin.

We would suggest the following theory to explain these phenomena: The charge distribution in γ-globulin is more asymmetric than in any other serum protein. γ-Globulin has therefore a very high dipole moment (1100 Debey units) and combines very easily with other proteins. In our experimental protein mixtures some degree of combination of γ-globulin and albumin must be expected. The binding power of such products will be small, and probably governed by the number of groups with dipole attraction (heteropolar cohesion type). As a result a certain proportion of the reactive groups of the γ-globulin will no longer be free to react with the reagents of the various tests, and the tendency towards a positive reaction becomes less and less (Table IV). The albumin molecule has ninety positive and ninety negative charges evenly distributed on the surface and the dipole moment is only 380 Debey units. The combining power is therefore small, and the influence of albumin on the results of the tests (from the point of view of inhibition) is negative. If the influence of albumin concentration relative to that of γ-globulin is compared, it is found to decrease in the following order: Takata > Cadmium > Thymol > Cephalin. Thus the effect of albumin is particularly strong in the Takata reaction, and we conclude that this test can only give a positive response when the serum protein contains both increased globulin and decreased albumin. The Takata reaction is more dependent on the albumin/globulin ratio than are the other three tests.

The lipoid-carrying proteins of serum are the α- and β-globulins and these have a particularly complex chemical structure. The lipoid plus carbohydrate content varies between 10 and 30 per cent (dry weight), and this seems to be the explanation for their ill-defined molecular weight. The molecular weight of β-globulin is estimated by Pederson (1945) from centrifugal studies as lying between 5×10^5 and $1{\cdot}9 \times 10^6$ (water of hydration included). Extraction of the lipoids brings the molecular weight down to the ordinary globulin value of *ca.* $1{\cdot}55 \times 10^5$. Pederson believes that the lipoids in the β-globulin molecule bind together several ordinary globulin molecules or even perhaps albumin molecules. If this is true it is probable that hepatitis serum, in which the content of lipoids, phospho-lipoids and carbohydrates is abnormally high, contains very large β-globulin molecules. We were unable to determine whether this was the case as no ultracentrifuge was available. The high lipoid content must reduce the hydrophilic properties of these lipoproteins to a considerable extent and thus increase the lability of the whole serum. From the point of view of the mechanism of the four tests this would mean that reagents which contain substances with lipotropic properties are more likely to react with sera containing increased lipoproteins. The reaction would depend on the mutual attraction of lipoid groups, and the subsequent binding would be of the homöopolar cohesion type. Such combinations need only small amounts of energy to disrupt them, and are consequently very vulnerable to changes in temperature. This probably explains why Cephalin tests must be kept at a constant temperature for the whole 24 hr. if comparable results are to be obtained. The inorganic reagent of

the cadmium test ($CdSO_4$) has no affinity for lipoid groups; in accord with this it is found (Table VII) that the Cephalin and Thymol tests still give a positive reaction at a dilution of hepatitis serum at which the cadmium reagent fails to show a response. At the greatest dilution tested (tube 31) 16 mg. per cent β-globulin plus 7 mg. per cent α-globulin and 25 mg. per cent γ-globulin are sufficient to give a positive reaction with the Cephalin and Thymol tests. Such extreme dilutions never occur in ordinary routine, but they give an indication of the sensitivity of the tests.

Finally it must be noted that the amount of additional albumin necessary to change a result from positive to negative seems to be extraordinarily high. We are inclined to believe that the effect of albumin on the general lability of a serum must be different *in vivo*. This may result from reciprocal interaction between albumin and lipoids, but we have not been able to reproduce this experimentally *in vitro*.

SUMMARY.

A comparison has been made of the results given with normal and hepatitis serum by the Thymol-turbidity test, the Cephalin-cholesterol-flocculation test, the Takata reaction and the Cadmium-turbidity test.

The effect of changing the composition of the serums by the gradual addition of pure γ-globulin and albumin has been investigated.

The relative response of the tests to sera containing increased γ-globulin or the lipoid-carrying α- and β-globulins is discussed from the point of view of the mechanism of the tests.

Electrophoretic patterns of all the components are given. They provide an exact quantitative basis for the experimental protein mixtures.

In general the Thymol, Cephalin and Cadmium tests give very similar results, but the Takata gives a smaller response.

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THE GROWTH OF *H. PERTUSSIS* ON MEDIA WITHOUT BLOOD.

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THE development of a vaccine to protect against whooping-cough has led to the need for an artificial medium which will grow *H. pertussis* satisfactorily. Little work has been done on the growth requirements of this organism, and the original medium first devised by Bordet and Gengou (1906; 1907) 41 years ago is still in general use with few modifications, although widely admitted to be far from ideal.

The modified Bordet-Gengou (B-G) medium most commonly used consists of 30 to 50 per cent whole blood and potato extract as well as a basis of peptone or tryptic digest of meat. Its composition suggests that the organism is highly fastidious; but even on this enriched medium single colonies do not reach countable size for 3 days, and on primary isolation growth may be even slower. Any attempt to simplify the medium has tended to cause a gradual change in the organism leading, after repeated subcultures, to loss of virulence and antigenicity, and change of phase from S to R. It is even possible that some loss of antigenic potency occurs very rapidly on the unsimplified B-G medium itself, leading to unsatisfactory batches of vaccine. Ability to grow on plain nutrient agar or in broth, a property which is usually only acquired after considerable training or laboratory storage, is invariably associated without complete loss of virulence and protective antigenicity for laboratory animals, as well as colonial and morphological changes which are more or less permanent (Lawson, 1939).

The nearest attempt to develop a synthetic medium for *H. pertussis* is that of Hornibrook (1939; 1940), who found that the required "Phase I" organism would grow in a mixture of amino-acids, salts and nicotinic acid and 0·1 per cent soluble starch. The main interest of his papers is that no growth would occur, even with a heavy inoculum, in the absence of starch or dextrin.

Our approach to the problem has been different from that of Hornibrook. Starting with a derived strain of *H. pertussis* that would grow well on ordinary 10 per cent blood agar, but not on plain nutrient agar or in broth, we have attempted to determine directly what factors in blood were responsible for its growth-promoting effect. It was discovered that a freshly isolated strain of *H. pertussis* (Phase I) could be trained to grow on 10 per cent blood agar as heavily as on the B-G medium, by two successive subcultures on starch-nutrient agar

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slopes containing diminishing quantities of blood. Repeated attempts to train the organism to dispense entirely with blood failed; and it was accepted as a suitable test strain for determining the role of blood in the growth of *H. pertussis*. Although the organism could not be regarded as typical "Phase I," it was considered to be a satisfactory indicator of the function of blood, because all attempts to induce it to grow without blood failed. Further detailed characteristics of this derived strain are given below.

METHODS.

1. *Media.*—The basal medium used throughout was a tryptic digest of beef. All blood fractions, except where otherwise stated, were human. Bordet-Gengou medium was prepared as described by Mackie and McCartney (1946).

2. *Organism.*—A derived, partially trained strain was obtained as follows: A freshly isolated Phase I strain of *H. pertussis* was inoculated from B-G medium on to a large agar slope of tryptic meat medium containing 0·1 per cent starch and 30 per cent blood. After 2 days' incubation at 35°, the growth, which was as good as on the original B-G medium, was subcultured on to a similar starch-agar slope containing 15 per cent blood, and the ensuing growth further subcultured on to a slope containing only 5 per cent blood. The organisms grew well after 5 days' incubation on this last medium, and were then found to grow on ordinary 10 per cent blood plates without starch as well as they did on the original B-G medium, even with small inocula. They were morphologically indistinguishable from the parent strain, and were agglutinated to titre by three different anti-Phase I sera. Absorption tests, however, with an anti-serum prepared from the parent strain showed that the derived strain had lost, completely or nearly completely, one of the antigens originally present. Virulence tests on the derived strain (undertaken by Mr. Proom) indicated a somewhat reduced virulence and toxigenicity for laboratory animals:

Route of injection.	M.L.D. for mouse (20 g.).	
	Normal Phase I.	Derived strain.
Intra-cerebral	50×10^6 .	50×10^6
Intra-nasal	25×10^6 .	25×10^8
Intra-peritoneal	15×10^8 .	25×10^8

The derived strain, although essentially Phase I or Smooth, must be regarded as having undergone slight degradation, the significance of which is not yet fully understood. It is subsequently referred to as "*Pertussis A.*" A large batch was immediately dried down by the method described by Stamp (1947), for use in growth experiments.

3. *Inoculation of media.*—The dried organisms were revived by growth for 3 or 4 days at 35° on a 10 per cent blood slope, which was then kept in the ice-chest to serve as inoculum-source. The inoculum itself was prepared from a 48 hours' growth on a blood slope inoculated from the first slope. The cells were scraped off with a wire and suspended in 2 ml. of tryptic meat broth to standard opacity (about 5×10^9/ml.), from which three 100-fold dilutions in broth were made. Actual inoculation was performed as soon as possible after dilution. A fresh ampoule of dried bacteria was used every fortnight. By these means it was hoped that stability of the culture would be maintained. As previously mentioned, there was no indication of any tendency to degradation, and attempts to train

the organism to grow in plain broth or on nutrient agar persistently failed, even with heavy inocula. Nevertheless, in all experiments, a plain broth or agar control was inoculated at the same time, in order to detect any possible sudden change. In experiments on solid media, 4 separate drop inocula (unspread), containing respectively 10^8, 10^6, 10^4 and 10^2 viable cells, were made on to each plate. All plates contained exactly 25 ml. of medium. The results were recorded as heaviness of confluent growth (judged roughly by eye) and diameter and numbers of colonies where discrete. In liquid media the inoculum was always about 100 cells inoculated into 5 ml. of medium in 50 ml. Erlenmeyer flasks and the growth was recorded in the usual way by numbers of plusses.

4. *Incubation.*—At 35° in air.

5. *Viable counts.*—On 10 per cent blood plates, using the method of Miles and Misra (1938).

6. *Protein content* of serum fractions was estimated by micro-Kjeldahl analysis of the N content.

RESULTS.

A. *Fractionation of blood.*

Because of the difficulty in determining growth in opaque liquid media, the initial experiments were all done on solid media, by the technique described. The results were clear cut and are not worth recording in full. It was found that the growth-promoting power of washed red cells, laked cells, serum or plasma

TABLE I.—*Growth of* Pertussis A *on Nutrient Agar Plates containing Various Fractions of Plasma.*

Medium	Dilutions of standard inoculum. 1 : 1	1 : 10^2	1 : 10^4	1 : 10^6
Control (tryptic meat agar alone)	Tr. +*	0	0	0
Plus—				
Dialysed plasma (2 per cent)	+++	+++	+++	100 (1 mm.)
Serum (2·2 per cent)	+++	+++	+++	100 (½ to 1 mm.)
Pure serum globulin (·07 per cent)	+*	0	0	0
Albumen residue (·07 per cent protein)	+++	+++	+++	100 (½ to 1 mm.)
Serum (2·2 per cent) + ·07 per cent additional globulin	+++	+++	+++	100 (½ to 1 mm.)
Horse serum albumen (·07 per cent)	+++	+++	+++	100 (1 mm.)
Whole blood (10 per cent)	+++	+++	+++	100 (1 mm.)

Growth for 5 days at 35°. +++ = heavy confluent growth; ++ = moderate growth; +, tr. + = slight growth; 0 = No growth.
Where colonies are discrete, their approximate number and size (diameter) are recorded.
* Probably no true growth (see remarks in text).

was as great as that of whole blood. Further fractionation was confined to plasma. The activity resisted prolonged dialysis and treatment at 56° for 30 min., but was completely destroyed by 30 min. in a boiling water bath. The diffusate after dialysis was quite inactive. The activity was clearly, therefore, associated with protein. By half-saturation of serum with ammonium sulphate, and two further precipitations and washings of the fraction thus isolated, serum globulin was extracted and found to be quite inactive, though not inhibitory ; the albumen residue, however, was as active as whole plasma. Some twice crystallized horse serum albumen was found to be as active as the human serum albumen residue. This horse serum albumen was dissolved in water and dialysed for 3 days in the ice-chest against 4 changes of 10 times its volume of distilled water ; it was then clarified by filtration through a paper pulp and finally sterilized by Seitz filtration. Table I shows the results given by these fractions. Little comment is necessary. The traces of growth recorded with the heaviest inoculum on the control plate and that containing only globulin do not probably signify actual multiplication, but simply the opacity of the inoculum itself. As will be shown later, not only does *Pertussis A* fail to multiply in plain broth or on agar ; the cells actually die off fairly rapidly.

It was now possible to carry out satisfactory growth experiments in liquid media. It was found that an inoculum of 100 viable cells would grow well in broth containing 0·1 per cent albumen. The initial growth turbidity was usually visible after 3 days' incubation, and heavy growth of about 5×10^9/ml. cells obtained after 5 days. The lowest concentration of albumen that would support growth varied with the batch of broth used, but was usually about 0·04 per cent. It was found essential to maintain efficient oxygenation by tubing the medium in small (5 ml.) lots in 50 ml. Erlenmeyer flasks. With a very heavy inoculum (about 10^8 cells) growth was first apparent after only 36 hours.

B. *Effect of charcoal.*

It seemed unlikely that the albumen was actually metabolized by the organism, and attention was drawn to the mysterious growth-promoting effect of charcoal on meningococci and gonococci reported by Glass and Kennett (1939). These workers found that charcoal (heated to 1000° to destroy organic matter) could replace blood in the cultivation of these bacteria on nutrient agar. They drew no conclusions, but put forward a number of tentative suggestions, amongst which was the possibility of a catalytic function due to iron present in the charcoal, and the alternative possibility that charcoal absorbed some inhibitory substance from the broth.

Activated charcoal (B.D.H.), made up in a 10 per cent suspension in water and autoclaved, was added to nutrient agar to a final concentration of 0·2 per cent. It was found to support the growth of *Pertussis A* as well as 0·04 per cent. albumen or 10 per cent blood (see Table II and Plate I, Fig. 1(*a*), (*b*) and (*c*)). Charcoal treated twice with boiling 12 per cent HCl for 20 min. (to remove any traces of iron) and subsequently washed was found to be just as efficient in supporting growth. In liquid media charcoal was, if anything, better than albumen ; the optimum concentration was found to be 0·2 per cent, although quite good growth would occur with 0·04 per cent. The growth of *Pertussis A* in charcoal broth was characteristic : the first sign of growth was a stickiness or pseudo-agglutination

TABLE II.—*Growth of* Pertussis A *on Nutrient Agar Plates with Added Charcoal, Starch Albumen or Whole Blood.*

Medium	Dilutions of standard inoculum. 1 : 1	1 : 10^2	1 : 10^4	1 : 10^6
Control (tryptic meat agar alone)	tr.	0	0	0
Plus—				
10 per cent whole blood	+++	+++	+++	200 (1 mm.)
0·4 per cent starch	+++	+++	s–c	125 ($\frac{1}{2}$ mm.)
0·2 per cent albumen	+++	+++	+++	200 ($\frac{1}{2}$–1 mm.)
0·2 per cent charcoal	+++	+++	+++	200 (1 mm.)

Explanation as in Table I. s–c = heavy semi-confluent growth.

of the fine charcoal fragments ; later the whole charcoal was glued together in a sticky, ropey mass which sank to the bottom, leaving a charcoal-free supernatant, turbid with growing organisms. On autoclaving, the glutinous material dissolved and the general appearance became that of the uninoculated medium. Glass and Kennett (1939) found that mere treatment of broth with charcoal, followed by its removal, did not produce a medium that would support growth of gonococci, and this phenomenon has been confirmed for *Pertussis A*. Moreover, charcoal-treated broth was found to support growth of *Pertussis A* if more charcoal was added, so that the failure of charcoal-treated broth alone cannot be ascribed to the previous absorption of essential growth factors as well. The charcoal must be present in the medium *during* growth.

c. *Effect of starch.*

Hornibrook (1939 ; 1940) and other workers have found the addition of starch necessary to semi-synthetic media in order to obtain growth of *H. pertussis ;* and 0·1 per cent starch has been successfully substituted for the potato extract in the B-G medium for the growth of the original strain. With *Pertussis A* itself, soluble starch (Analar) in concentrations from 0·04 per cent to 0·4 per cent in solid media acted similarly, though not quite so efficiently or reliably (with small inocula) as charcoal or albumen (Table II). In liquid media (in concentrations from 0·1 to 0·5 per cent) and with small inocula of 100 cells growth was most unreliable.

D. *Activity through cellophane.*

Hornibrook (1939) has stated that starch or serum was necessary for the growth of *H. pertussis* in his semi-synthetic medium, and that the serum could exert its effect even though retained in a cellophane sac suspended in the medium. This of course might have been due to diffusible substances present in the serum, but Dubos (1946) has reported that serum albumen itself could exert its growth-promoting effect on the tubercle bacillus, although confined within a cellophane sac. Accordingly it was decided to find out whether albumen would support the growth of *Pertussis A* under similar conditions. Two ml. of 1·5 per cent crystalline horse serum albumen was suspended, within a cellophane sac, in 10 ml.

of broth which was inoculated (outside the sac) with 5×10^5 cells of *Pertussis A*. Growth was first visible after 5 days' incubation. The same amount of albumen added direct to the medium gave visible growth with the same inoculum in 2 days. This effect was confirmed by laying circles of sterile cellophane on the surface of charcoal-agar, albumen-agar and blood-agar plates and inoculating the centre of the exposed surface. Growth occurred almost as well as in the absence of cellophane, though slightly delayed, and single colonies developed satisfactorily (Fig. 2). It need hardly be pointed out that neither charcoal nor albumen could possibly pass through cellophane, and it is quite clear that these two substances exert part, at least, of their effect by absorbing some toxic material from the medium.

E. *Effect of fatty acids* on growth.*

The main question that remained to be decided was the nature of the toxic material thus removed from broth by charcoal and albumen. Davis and Dubos (1946) ascribed the stimulating effect of albumen for the tubercle bacillus to its power of combining with the minute quantities of oleic acid present in Dubos' "Tween 80" medium. Oleic acid, they found, was very highly inhibitory to the tubercle bacillus. Long chain fatty acids and their soaps, especially unsaturated acids, have long been known to be highly bactericidal to a variety of micro-organisms (Kodicek and Worden, 1945). It is also well known that their action is counteracted by serum (Lamar, 1911; Walker, 1924; Eggerth, 1927). Charcoal, moreover, is known to absorb fatty acids firmly (Klaesson, 1946); and the same is true of starch (Schoch and Williams, 1944). The inhibitory effect of soaps on pneumococci (Walker, 1924), haemolytic streptococci (Burtenshaw, 1942), meningococci and gonococci (Walker, 1926), *C. diphtheriae* (Walker, 1925; Burtenshaw, 1942), *Erysipelothrix rhusiopathiae* (Hutner, 1942; Kodicek and Worden, 1945) and other organisms has been described, but no information exists concerning *H. pertussis*.

DESCRIPTION OF PLATE.

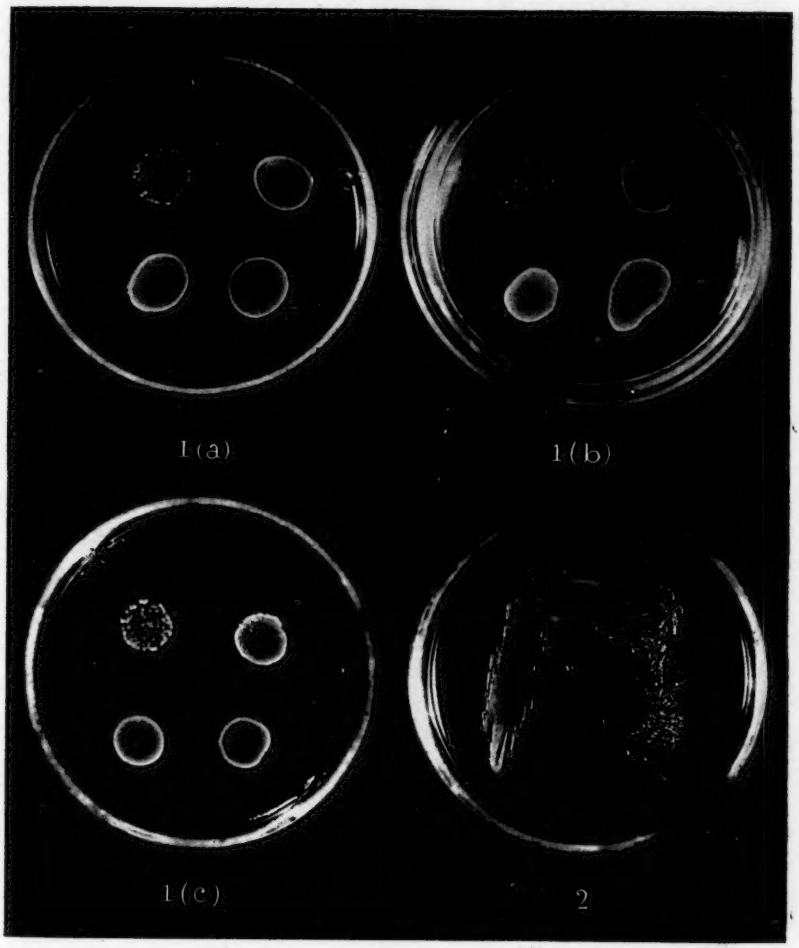
FIG. 1.—Comparison of the growth of *Pertussis A* on blood, albumen and charcoal in a basal medium of tryptic meat agar. 4 drop-inocula were made on to each plate. The drop with discrete colonies contained 100 cells, the others, 10^4, 10^6, 10^8 cells respectively, passing clockwise. (*a*) 10 per cent blood. (*b*) 0·04 per cent horse serum albumen. (*c*) 0·2 per cent charcoal.

FIG. 2.—Growth of *Pertussis A* (from inoculum spread by wire) on the surface of cellophane lying on 0·2 per cent charcoal in nutrient agar.

Effect of certain inhibitory substances on the growth of *Pertussis A*, as demonstrated by the "Albumen Cup" method (Text and Table III).

FIG. 3.—Control.
FIG. 4.—4 μg./ml. oleate.
FIG. 5.—4 μg./ml. linoleate.
FIG. 6.—4 μg./ml. linolenate.
FIG. 7.—4 μg./ml. palmitate.
FIG. 8.—2 mg./ml. extract from charcoal used for growth of *Pertussis A* in broth.

* Throughout this work the words "fatty acid" and "soap" have been used interchangeably. All experiments have been done in relatively well buffered tryptic meat media of pH 7·6. At this pH the proportion of free fatty acid will be very small, and in any case, at the low concentrations used, will not be affected by whether it is added as such or in the form of a soap.



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A systematic investigation on the susceptibility of *Pertussis A* to soaps was undertaken with pure samples of oleic, linoleic, linolenic, palmitic and stearic acids (used as their sodium salts).

On the hypothesis that the toxic action of fatty acids is antagonized by albumen, their inhibitory effect might be expected to be masked unless the concentration of albumen was kept sufficiently low. With too little albumen, on the other hand, there would be no growth at all, even without the addition of fatty acid. The balance might therefore be expected to be rather critical. To obviate the complication of having to titrate many different concentrations, it was decided to do all preliminary work on solid media by means of a special technique.

The substance to be tested was incorporated in the agar plates (final vol. 25 ml.). One ml. of a suspension of *Pertussis A* (containing approx. 10^9 organisms /ml.) was flooded over the plate and the excess pipetted away. Three porcelain cups (as used and described by Heatley (1944) for penicillin assay) were sealed on the surface, and into these cups were added, respectively, 1, 2 and 3 standard drops of horse serum albumen solution and the plates incubated for 4 days. The albumen diffused slowly out into the medium, producing a gradient with a limiting concentration necessary for growth of *Pertussis A* at a distance from the centre of the cups depending, amongst other factors, upon the degree of inhibition by the substance under test. Growth plaques thus developed around the cups (Fig. 3), the diameter of which depended only (other conditions being equal) upon the amount of albumen in the cup and the degree of anti-bacterial action of the test material. As might have been expected, different batches of nutrient agar gave very different sized growth plaques, even with identical inocula. This emphasized the necessity of using the same batch of basal medium throughout any one experiment, and suggested that tryptic meat medium, as ordinarily prepared, contained very varying quantities of the toxic material removable by albumen or charcoal.

The method is, in fact, the ordinary penicillin cup test in reverse, with the inhibitor in the medium instead of within the cups. In practice, it was found to give approximate but reliable and reproducible results. The main advantages were :

(*a*) Ability to demonstrate the inhibitory effect of a fatty acid or similar substance without the need to control the albumen concentration.

(*b*) A reduction to a minimum (in solid media) of diffusion effects.

(*c*) Suitability for the estimation of the antibacterial action of unknown toxic substances in crude extracts.

(*d*) The rapid and simple technique, saving much time and material.

A reduction in size of the growth plaque is presumably due to a combination of (1) the anti-albumen effect of the substance tested, so that there is less "free" albumen at the periphery to exert its activity, and (2) a direct lethal action of the substance on the cells before the albumen reaches them, accelerating the death rate so that even if there is enough "free" albumen present to exert its effect no growth results. The factors concerned are no doubt complex, but the net result is that a general antibacterial effect is detected with considerable sensitivity.

The results, in which recorded figures are the diameters of growth plaques (in mm.) developing around the cups after 4 days' incubation, are summarized

in Tables III and IV and illustrated in Fig. 3 to 8. Results with the unsaturated fatty acids were confirmed in experiments with liquid media (Table V).

TABLE III.—*Effect of Soaps on the Growth of* Pertussis A *on Nutrient Agar Plates. "Albumen-cup" Method (see text).*

Soap.	μg./ml.	Diameter of growth plaques (mm.) around cups containing the following quantities of albumen:		
		1 drop.	2 drops.	3 drops.
—	0	15	18	20
Sod. oleate	8	12	12·5	13·5
Sod. linoleate	8	(8)	10	11
Sod. linolenate	8	(8)	(8)	10·5
Sod. palmitate	8	16	20	19
Sod. palmitate	40	25	21	25
Sod. stearate	8	18	21	24
Sod. stearate	40	27	27	35

The diameter of the cups is 8 mm., so that a growth plaque of less than 9 mm. is not measurable and is recorded as (8).

TABLE IV.—*Titration of the Inhibitory Effect of Different Concentrations of Sod. Oleate on the Growth of* Pertussis A *on Nutrient Agar. "Albumen-cup" Method.*

Conc. (μg./ml.) of sod. oleate in nutrient agar.	Diameter of growth plaques (mm.) around cups, containing the following quantities of albumen:		
	1 drop.	2 drops.	3 drops.
0	15	17	19
0·8	15	17	19
2·0	13	15	16
4·0	13	15	16
10	10	13	14
20	10	10	12
40	(8)	(8)	9

For full explanation see text and footnote to Table III.

It is clear that—

(*a*) The unsaturated fatty acids (oleic, linoleic, and linolenic) are highly inhibitory at a concentration as low as 4 μg./ml. The two saturated fatty acids (palmitic and stearic), on the other hand, have no action at 8 μg./ml.; at the higher concentration of 40 μg./ml. they actually appear to stimulate growth (Table III).

(*b*) The inhibitory effect of the unsaturated soaps is completely counteracted by albumen. For example, in the presence of 8 μg./ml. oleate no growth occurred at an albumen concentration of 0·06 per cent., but with 0·24 per cent albumen growth was practically as good as without any oleate at all (Table V).

TABLE V.—*Effect of Certain Soaps and Albumen on the Growth of* Pertussis A *in Broth. Inoculum* : 100 *Organisms.*

Soap.	ml. 1·5 per cent albumen (in 5 ml.).	Growth. 3 days.	Growth. 5 days.
Nil	0	—	—
	0·1	—	++
	0·2	—	+++
	0·4	—	+++
	0·8	tr.	+++±
Oleate	4 μg./ml. 0	—	—
	4 μg./ml. 0·1	—	—
	4 μg./ml. 0·2	—	+
	4 μg./ml. 0·4	—	+++
	4 μg./ml. 0·8	tr.	+++
	8 μg./ml. 0	—	—
	8 μg./ml. 0·1	—	—
	8 μg./ml. 0·2	—	—
	8 μg./ml. 0·4	—	++±
	8 μg./ml. 0·8	—	+++
Linoleate	4 μg./ml. 0	—	—
	4 μg./ml. 0·1	—	—
	4 μg./ml. 0·2	—	+++±
	4 μg./ml. 0·4	tr.	+++±
	4 μg./ml. 0·8	+±	++++
	8 mg./ml. 0	—	—
	8 mg./ml. 0·1	—	—
	8 mg./ml. 0·2	—	—
	8 mg./ml. 0·4	tr. +	++++
	8 mg./ml. 0·8	++	++++
Linolenate	4 μg./ml. 0	—	—
	4 μg./ml. 0·1	—	—
	4 μg./ml. 0·2	—	++±
	4 μg./ml. 0·4	tr.	+++
	4 μg./ml. 0·8	tr. +	+++±
	8 μg./ml. 0	—	—
	8 μg./ml. 0·1	—	—
	8 μg./ml. 0·2	—	tr.
	8 μg./ml. 0·4	—	+++
	8 μg./ml. 0·8	tr.	++++

The sensitivity of *Pertussis A* to unsaturated fatty acids and the known property of charcoal and albumen in absorbing these substances supports the hypothesis that they, or related compounds, might be the main components of the toxic material developed in meat broth. It is highly probable that both

saturated and unsaturated soaps occur in most " natural " media, though their content is likely to be very variable.

F. *Extraction of toxic material from broth.*

It remained to demonstrate directly the presence of a toxic substance in tryptic meat broth. 500 ml. of broth containing 1 g. of charcoal were distributed in 10 Roux flasks, and inoculated with *Pertussis A*. After 6 days' incubation at 35° the bottles were autoclaved, and the charcoal was filtered off and extracted, without washing, with 100 ml. of methanol in a Soxhlet for 3 hours. The extract was evaporated down *in vacuo*, leaving 2.03 g. of a yellow fatty material which dissolved easily in 20 ml. of broth to form a stable cloudy emulsion. It was tested for anti-*Pertussis* action by the albumen cup method and found to give marked inhibition at a concentration of 1 in 5000, corresponding very approximately to 1/100 of the activity of pure oleate (Table VI).

TABLE VI.—*Titration of the Inhibitory Effect of Toxic Material Extracted from Charcoal Used for Promoting Growth of* Pertussis A *in Broth. "Albumen-cup" Method.*

Conc. of extract in nutrient agar (μg./ml.).	Diameter of growth plaques (in mm.) around cups containing the following quantities of albumen :		
	1 drop.	2 drops.	3 drops.
Nil (control). .	10·5	13	14
40 . . .	(8)	11·5	12·5
200 . . .	(8)	(8)	11
600 . . .	(8)	(8)	(8)

For full explanation of method see text and footnote in Table III.

These results confirm conclusively that charcoal removes from broth cultures material that is highly toxic to *Pertussis A*.

G. *Viability of* Pertussis A *in plain broth.*

It seemed important at this stage to obtain some idea of the survival time of the organism in plain broth and on agar without the addition of albumen and charcoal.

Ten ml. of broth were inoculated with approximately 25,000 cells and incubated at 35°, samples being removed at intervals for viable counts (Table (VII (*a*)). It is clear that all organisms were dead within 20 hours. This was confirmed by a similar experiment with an inoculum of only 100 cells in a series of flasks; instead of attempting viable counts, 0·5 ml. of 2 per cent albumen was added after intervals to each flask in order to determine at what stage growth failed to occur. Table VII (*b*) shows that some cells were still viable after 22 hours, but after 30 hours all were dead.

With heavy inocula on the surface of agar plates it can be shown, using the albumen cup technique already described (modified only in that the same quantity of albumen was added to each cup in succession at different intervals after inoculation) that even a large inoculum does not survive longer than 46 hours without albumen (Table VII (*c*)).

TABLE VII.—*Viability of* Pertussis A *in Plain Broth and on Nutrient Agar, without Albumen.*

(a) *Viable counts in broth.*

	Hours after inoculation.					
	0	2	4½	7	23	31
Viable cells/ml. . .	1820	1330	1270	780	0	0

Inoculum : Approx. 25,000 cells into 10 ml.

(b) *Effect of adding albumen to broth at different periods after inoculation.*

Time of adding albumen, hours after inoculation.	Subsequent growth after 7 days' incubation.
0	+
2	+
4	+
6	+
8	+
22	+ (delayed)
30	0

Inoculum : 100 cells into 4·5 ml. of broth.
Albumen added : 0·5 ml. of 1·5 per cent solution.

(c) *Effect on size of growth plaque on agar (in the albumen-cup technique) of adding albumen to the cups at varying intervals after inoculation.*

Time of adding 3 drops of 2 per cent albumen to cups hours after inoculation.	Diameter (in mm.) of growth plaque after 7 days' incubation.
0	22·5
6	18
22	16
30	13·5
46	0

It can therefore be concluded that cells of *Pertussis A* are killed fairly rapidly if incubated in broth or on agar without the addition of some suitable protective colloid.

DISCUSSION.

It has been shown that charcoal, albumen, probably starch and presumably blood owe their growth-promoting effect in broth, in part at least, to their power of absorbing a substance or substances demonstrably toxic to *Pertussis A*. The evidence so far suggests that these toxic substances are unsaturated fatty acids or nearly related compounds. The chemical nature of the toxic extract from charcoal is being further investigated, and it is hoped that the active fraction will be purified and identified. It is, however, far from certain (*a*) whether the whole of the growth-promoting power of charcoal or albumen can be ascribed to

this property of fatty acid absorption, and (*b*) whether all the toxic substances are originally present in the broth or are formed, to some extent at least, during the growth of the organism. The fact that mere treatment of broth with charcoal (followed by its removal) was not sufficient to promote growth suggests that a fraction of the toxic material may result from the metabolism of the cells themselves.

It is likely that these findings will prove to have a wider significance and apply to other genera of bacteria, especially to those that are known both to need the addition of serum to ordinary media for good growth, and to be susceptible to fatty acids. Both meningococci and gonococci are very sensitive to fatty acids (Walker, 1926), and the growth-promoting effect of charcoal discovered by Glass and Kennett (1939) is, in the light of the present work, almost certainly due largely to its property of combining with toxic fatty acids or similar substances in the culture, as with *Pertussis A*. That some such process is responsible is also suggested by the work of Gould, Kane, and Mueller (1943), who found that starch was necessary for the growth of gonococci on a solid synthetic medium, and concluded that the function of the starch was to neutralize the inhibitory effect of some batches of agar. Later, Ley and Mueller (1946) isolated from agar, by methanol extraction, a product similar to the higher fatty acids, which was inhibitory to gonococci and was neutralized by starch. In liquid synthetic media, however, starch was found to have no differential effect.

The extreme susceptibility of pneumococci to unsaturated fatty acids may well have some connection with the growth-promoting power of serum for this organism; and the same principles may apply to streptococci, corynebacteria and other organisms. It has, in fact, been somewhat of a mystery why so many micro-organisms need, or thrive on, plasma or serum. The tacit assumption has usually been that such organisms require some growth factor present in plasma that is absent from broth. The alternative explanation—that broth may contain too much of something, rather than too little—has received small attention.

SUMMARY.

1. Some of the growth requirements of a derived strain of *H. pertussis* that would grow on 10 per cent blood agar, but not on plain tryptic meat agar, have been investigated.

2. The active fraction in blood was found to be albumen. Charcoal could also completely replace blood.

3. The growth-promoting effects of albumen and charcoal could be demonstrated through cellophane.

4. If incubated in broth without charcoal or albumen, the organisms died rapidly.

5. The inhibitory effect of higher unsaturated fatty acids upon *H. pertussis* was demonstrated, and the hypothesis put forward that the growth-promoting power of both albumen and charcoal is attributable, in part at least, to their ability to combine with toxic fatty acids (or similar compounds) present in the culture.

6. By methanol extraction of charcoal used for the growth of *H. pertussis* in broth, a material has been obtained which is highly toxic to the organism.

7. The implications and wider significance of these facts are discussed, with

particular reference to the cultivation of organisms already known to need serum for growth and to be susceptible to the toxic effects of fatty acids.

My thanks are due to Sir Paul Fildes, F.R.S., at whose instance this work was begun, for his advice and encouragement, and to other members of the Medical Research Council's Unit for Bacterial Chemistry for helpful suggestions; to Dr. R. Cruickshank, Director of the Central Public Health Laboratory at Colindale, for the original culture of *H. pertussis*; to Mr. H. Proom, Bacteriologist at the Wellcome Physiological Research Laboratories, for virulence tests on the derived strain of *H. pertussis* used in these experiments; to Dr. W. T. J. Morgan, of the Lister Institute, for gifts of pure fatty acids; to Dr. R. A. Kekwick, of the Lister Institute, for the gift of some crystalline horse serum albumen; to L. J. Hale for taking the photographs; and to S. V. Peacock and Miss A. H. Hearle for technical assistance.

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